DOES NOT CIRCULATE

UNIVERSITY OF MICHIGAN
SEP 5 1952
MEDICAL LIBRARY

CANCER RESEARCH

VOL. 12 AUGUST 1952 No. 8

CONTENTS

SYMPOSIUM ON IMMUNOGENETICS AND CARCINOGENESIS

Morris K. Barrett. Some Immunogenetic Influences upon Transplanted Tumors 535
George D. Snell. The Immunogenetics of Tumor Transplantation 543
Elizabeth C. Miller and James A. Miller. *In Vivo* Combinations between Carcinogens and Tissue Constituents and Their Possible Role in Carcinogenesis 547
Hugh J. Creech. Chemical and Immunological Properties of Carcinogen-Protein Conjugates . . 557

Emery M. Gal, Fung-Haan Fung, and David M. Greenberg. Studies on the Biological Action of Malononitriles. I. The Effect of Substituted Malononitriles on the Growth of Transplanted Tumors in Mice 565
N. L. Petrakis, H. R. Bierman, and M. B. Shimkin. Addendum: Substituted Malononitriles in Neoplastic Diseases in Man 573
Emery M. Gal, Fung-Haan Fung, and David M. Greenberg. Studies on the Biological Action of Malononitriles. II. Distribution of Rhodanese (Transulfurase) in the Tissues of Normal and Tumor-bearing Animals and the Effect of Malononitriles Thereon 574
Claude Allard, Gaston de Lamirande, and Antonio Cantero. Mitochondrial Population of Mammalian Cells. II. Variation in the Mitochondrial Population of the Average Rat Liver Cell during Regeneration. Use of the Mitochondrion as a Unit of Measurement 580
S. Albert and Ralph M. Johnson. The Effect of Inherited Factors, the Milk Agent, and Physiological State on Phosphorus Metabolism of Endocrine Glands of Mice 584
George Klein. The Nature of Mammalian Lymphosarcoma Transmission by Isolated Chromatin Fractions 589
Sidney D. Kobernick, Edna W. Toovey, and D. R. Webster. Effects of the Application of Carcinogens to Exposed Gastric Mucosa in the Rat 591
John J. Bittner. Studies on the Inherited Susceptibility and Inherited Hormonal Influence in the Genesis of Mammary Cancer in Mice 594
Committee on Standardized Nomenclature for Inbred Strains of Mice. Standardized Nomenclature for Inbred Strains of Mice 602
Announcements 614

THE OFFICIAL ORGAN OF THE
AMERICAN ASSOCIATION FOR CANCER RESEARCH, INC.
Published by THE UNIVERSITY OF CHICAGO PRESS

CANCER RESEARCH

This journal is sponsored by The American Association for Cancer Research, Inc.; The Anna Fuller Fund; The Jane Coffin Childs Memorial Fund for Medical Research; The Elsa U. Pardee Foundation; and The American Cancer Society.

HAROLD P. RUSCH, *Editor-in-Chief*
ELIZABETH B. POHLE, *Technical Editor*

Editorial Advisory Board

JOSEPH C. AUB
JOHN J. BITTNER
AUSTIN M. BRUES
DALE R. COMAN
E. V. COWDRY
HUGH J. CREECH
M. DEMEREC
F. DURAN-REYNALS
JACOB FURTH
W. U. GARDNER
ALFRED GELLHORN
SAMUEL GRAFF
HARRY S. N. GREENE
JESSE P. GREENSTEIN
ERICH HIRSCHBERG
CHARLES HUGGINS
ARTHUR KIRSCHBAUM
ALBERT L. LEHNINGER
C. C. LITTLE
BALDUIN LUCKÉ
G. BURROUGHS MIDER
JAMES A. MILLER
IRA T. NATHANSON
C. P. RHOADS
A. J. RIKER
MICHAEL B. SHIMKIN
PAUL E. STEINER
C. CHESTER STOCK
ROBERT E. STOWELL
ALBERT TANNENBAUM
SHIELDS WARREN
ARNOLD D. WELCH

NOTICE TO MEMBERS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH

Officers for 1952–53

President: STANLEY P. REIMANN, Lankenau Hospital Research Institute, Philadelphia 11, Pa.
Vice-President: HAROLD P. RUSCH, University of Wisconsin, Madison 6, Wis.
Secretary-Treasurer: HUGH J. CREECH, Institute for Cancer Research, Fox Chase, Philadelphia 11, Pa.

Changes of address of members of the American Association for Cancer Research should be sent promptly to Dr. Creech for recording in the files of the Association and also to insure receipt of the journal *CANCER RESEARCH* by active members of the Association.

CANCER RESEARCH is published monthly for Cancer Research, Inc., by the University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois. Subscriptions are by volume only and are payable in advance. The subscription price is $12.50 per volume; the price of single issues is $1.25. Orders for less than a full volume will be charged at the single-issue rate. Postage is prepaid by the publishers on all orders from the United States and its possessions. No extra charge is made for countries in the Pan American Postal Union. Postage is charged extra as follows: For Canada and Newfoundland, 50 cents per volume (total $13.00), 5 cents per issue (total $1.30); for all other countries in the Postal Union, $1.00 per volume (total $13.50), 10 cents per issue (total $1.35).

The following is an authorized agent:

For the British Empire, except North America and Australasia:

Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, England. Prices of subscriptions and of single copies may be had on application.

Business communications, remittances (in United States currency or its equivalent), and subscriptions should be addressed to THE UNIVERSITY OF CHICAGO PRESS, 5750 Ellis Avenue, Chicago 37, Illinois. All other communications should be addressed to Harold P. Rusch, M.D., McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wisconsin.

Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

No responsibility is accepted by the Editors, by Cancer Research, Inc., or by the publishers of *CANCER RESEARCH* for opinions expressed by contributors.

NOTICE TO SUBSCRIBERS: If you change your address, please notify us and your local postmaster immediately.

Entered as second-class matter, February 15, 1949, at the post office at Chicago, Ill., under the Act of March 3, 1879.

Acceptance for mailing at special rate of postage provided for in United States Postal Act of October 3, 1917, Section 1103, amended February 28, 1925, authorized June 1, 1950.

Copyright 1952 by Cancer Research, Inc.

CANCER RESEARCH

VOLUME 12 AUGUST 1952 NUMBER 8

Symposium
Immunogenetics and Carcinogenesis

(The following four papers by M. K. Barrett, George D. Snell, Elizabeth C. and James A. Miller, and Hugh J. Creech were presented in New York on April 13, 1952, before the 43d Annual Meeting of the American Association for Cancer Research, Inc.)

Some Immunogenetic Influences upon Transplanted Tumors

MORRIS K. BARRETT

(National Cancer Institute, National Institutes of Health, Bethesda, Md.)

It is a pleasure to be here this morning and to be permitted to participate in this first Symposium on the subject of "Immunogenetics." I was glad to hear the Chairman say that he had never before heard the word "Immunogenetics," because I too, had not seen this word until I saw it in communications from the program committee.[1] At first sight, however, the word appears to be a useful one. Many people have been aware for years that the sciences of immunity and genetics do impinge upon each other at certain points. Without going deeply into the matter, this is evidenced by the fact that workers in immunology have spoken of the genetic aspects of the blood groups (10), of the immunologic relationships between species (12), and Cumley (8) has more recently worked upon the inheritance of specific precipitable proteins in dove hybrids. Geneticists, too, have been conscious of the overlap in these fields, and this may be seen clearly in many discussions of the transplantation of tissues and tumors (11, 13). It, therefore, seems possible that this hybrid word may be a desirable addition to our vocabulary, at least until more is known of the overlapping areas of the two sciences.

[1] Since this remark was made, I have found that Irwin used the term "immunogenetics" as early as 1936, and more recently it was included in the title of an article by R. W. Cumley and M. R. Irwin, in Genetics, 27:177–79, 1942. However, I have not yet found a definition for the term.

If one who is neither an immunologist nor a geneticist may assume the privilege of defining the word "Immunogenetics," I propose that, for the time being, it be rather loosely defined as referring to those areas in which the disciplines of genetics and immunology impinge upon or overlap each other by reason of concept, technics, or knowledge. In this loose definition, we will be able to find sufficient room for changing concepts until future knowledge makes it possible to define the word more closely.

Because the subject is extensive and time is short, I shall not attempt to review the literature of this field. Instead, I shall try to develop a few concepts and raise some questions by presenting some of our own work with occasional mention of the work of others. I am aware that in doing this I shall undoubtedly fail to mention many previous workers who have contributed to knowledge in this field, and I apologize to all such.

For many years I have been working in an area of cancer research which seemed to be both genetic and immunologic in character. This began with some work on induced resistance to tumors which is illustrated by Chart 1 (3). There is a great deal to be seen in this figure, and there is not sufficient time at present to go into all the details. I hope that you will not be confused by the amount of material shown, but instead will allow me to call

your attention to certain aspects of these results. The hosts were of three kinds, Rockefeller Institute (a nonhomozygous strain), strain A, and strain C57BL, both of which are homozygous. For most of the work tumor 15091A was used. This is a well known tumor which originated in the Jackson Memorial Laboratory in a strain A female. I have also used Bashford 63 which typifies a widely transplantable tumor of unknown origin and may be looked upon as genetically unknown or indifferent. One set of experiments was done with tumor 755 which, as you know, originated in a strain C57BL mouse in the laboratories of Dr. Bagg. All three tumors are mammary carcinomas. Nonspecific resistance was induced in the hosts by the injection of defibrinated blood; 0.2 cc. of blood was injected under the dorsal skin of the mouse, and an incubation period of 10 days was allowed to elapse. We later came to believe that 21 days is a better period and have adopted this for subsequent work. At the end of the incubation period the mice were inoculated in the right inguinal region, at a distance from the site where the blood had been injected, with one or the other of these tumors. The mice were inspected at weekly intervals, and the presence or absence of a tumor was noted and its size measured with calipers. In this work we did not examine for lethal tumors, and these results all indicate what are commonly referred to as "takes." You will observe that, by the proper selection of materials, one may obtain a whole spectrum of results in which the percentage of "takes" ranges from 100 per cent down to as little as 5 per cent, and almost any percentage can be found between these extremes. The actual percentage of "takes" that is obtained depends upon the genetic interrelationships of the mouse, the blood injected, and the tumor. For tumor 15091A in Rockefeller mice the controls had 100 per cent takes, whereas those mice injected with strain A blood had only 7 per cent takes. In other words, the mice previously injected with blood were highly resistant to the tumor. If the same sort of mice, injected with the same sort of blood, were inoculated with Bashford 63, qualitatively similar results were obtained, but they differed quantitatively from those obtained with the previous tumor. So throughout the work various degrees of immunity were obtained, with

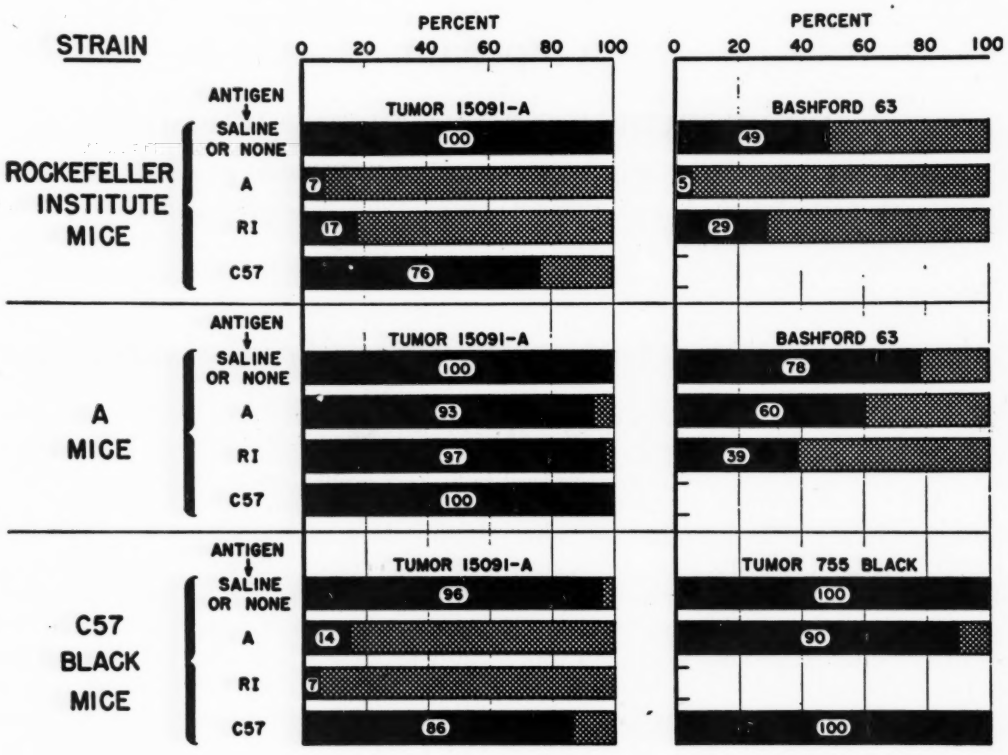


CHART 1.—Summary of data from some experiments in which resistance to transplantation of tumors was induced by the prior inoculation of homologous blood. The black areas represent the percentage of "takes." All hosts in each of the three rows were of the strain indicated at the left. All hosts in a line were immunized with the blood indicated at the left of that line. The tumor inoculated is shown at the top of each block. None of the differences in the block showing strain A tumors in strain A mice or strain C57 black tumors in strain C57 black is statistically significant. All other differences are significant except those between 7 and 17 in the upper left, 7 and 14 in the lower left, and 86 and 96 in the lower left blocks. A total of 768 mice is represented on the chart.

varied materials; but your attention should be called to the fact that, when the strain A tumor was inoculated into strain A mice, none of the animals were resistant. In other words, no resistance could be induced in a mouse against a tumor which originated in its own strain. This result is confirmed in the work with the strain C57BL mice where no resistance was obtained against the C57BL tumor; but resistance could be obtained against the A tumor, the degree depending upon the blood injected. (Detailed data can be found in [3]).

There are some interesting things about the specificity observed in this work. We have just seen that there is a specificity which relates the source of the materials one to the other. There is also a specificity that may be of a somewhat different kind, which can be seen when certain other tumors are used. It was observed that Rockefeller Institute mice could be made highly resistant to tumor 15091A by the injection of strain A blood (30 hosts, two tumors) and highly resistant under the same circumstances against Bashford 63 (44 hosts, two tumors). However, under the same conditions these mice are not resistant to Sarcoma 180 (twenty hosts, twenty tumors). In other words, here may be a specificity of the tumor which cannot be stated in the simple genetic terms previously used. A genetically known tumor was susceptible to this resistance. Another tumor that is genetically unknown and heterozygous was also susceptible to this resistance, but Sarcoma 180, also genetically unknown and heterozygous, was not susceptible to this resistance. Similar results have been obtained in strain C (BALB/c) mice. Such mice are susceptible to Sarcoma DBA 49 (50 hosts, 46 tumors), but after being immunized by strain DBA blood they became highly resistant against the sarcoma which originated in strain DBA (50 hosts, three tumors) but not against Sarcoma 37 (thirteen hosts, eleven tumors). While the number of animals tested with Sarcoma 37 is not large, the results obtained for the two tumors are significantly different. It is also noteworthy that the strain DBA tumor is a sarcoma. It was not previously known with certainty that sarcomas might be as susceptible to this nonspecific type of resistance as are carcinomas. Another aspect of this field may be seen by injecting strain I blood into either strain C or strain Y mice. This was done because there is an oft recurrent question as to whether some humoral material in the injected blood is specifically antagonistic to these tumors. From the work of Andervont (2), it is known that strain I mice are comparatively resistant to Sarcoma 37 and that the tumor often regresses when implanted intracutaneously. On the other hand, strains Y and C are highly susceptible to Sarcoma 37. For that reason strain I blood was injected into strain C and strain Y mice, and the recipients were then tested for resistance to Sarcoma 37. They were entirely nonresistant (nine strain C hosts, nine tumors; 32 strain Y hosts, 32 tumors), and nothing appeared to be transferred in the strain I blood which would make these animals resistant to Sarcoma 37. A consideration of dosage indicates that this result should be taken as conclusive only in comparison with the other results shown. It is possible that a strain Y mouse might be made resistant to Sarcoma 37 by transferring all or most of the blood of a strain I mouse, something that we have not done.

TABLE 1

PROPORTION OF SUCCESSFUL TRANSPLANTS OF SARCOMA DBA 49 INTO MICE OF STRAINS DBA AND C AFTER A PRIOR INJECTION OF EITHER SALINE OR BLOOD OF THE ALTERNATE STRAIN

The numerators represent the number of "takes" at 3 weeks; the denominators represent the total number of mice inoculated. The difference between the 2 groups of strain C mice is highly significant statistically; $P < 0.001$.

STRAIN DBA HOSTS		STRAIN C HOSTS	
After saline	After C blood	After saline	After DBA blood
76/76	14/14	8/10	0/10
	18/18	15/16	0/16
	6/6	10/10	1/10
	13/13	11/14	2/14
76/76	51/51	44/50	3/50
100 per cent	100 per cent	88 per cent	6 per cent

I have already referred to Sarcoma DBA 49, an unusual tumor and a highly desirable one for this type of work. We obtained it from Dr. Andervont, in whose laboratory it arose spontaneously in a strain DBA female. It is unusual in that it not only grows in all mice of the strain of origin, but also grows progressively in 90 per cent of strain C BALB/c) mice. Such a tumor made it possible to keep very close genetic control of the materials while carrying these investigations a few steps further (6). In order to be sure that this sarcoma was susceptible to this type of resistance, the experiments of Table 1 were done. It is obvious from the results that strain C mice can be made highly resistant against this tumor, since the tumor grew in 88 per cent of the control strain C mice, whereas only 6 per cent of the strain C mice were susceptible to the tumor after the injection of strain DBA blood. The converse relationship is not true, and it is again seen that these DBA mice could not be made resistant against a tumor which originated in the same strain. We again wished to test certain other

specificities of this reaction, and some experiments along the lines of the earlier work with Sarcomas 37 and 180 are illustrated in Table 2 (which includes only mice in which the primary inoculation grew). When strain DBA mice were inoculated in the tail with strain DBA sarcoma, all the mice, or nearly all, grew the tumor. The tail is an unfavorable inoculation site, and more difficulty was experienced with strain C hosts; only a small percentage grew the tumor in the tail. In accordance with the method of Andervont (1), the tumor-bearing tails of both groups were subsequently amputated, and 1 week later a secondary inoculation of the same tumor was made in the groin.

TABLE 2

SUCCESSFUL INOCULATIONS OF SARCOMAS DBA 49 OR 37 INTO THE GROIN OF MICE 1 WEEK FOLLOWING THE EXCISION OF SARCOMA DBA 49 GROWING IN THE TAIL

Numerators show the number of tumors present at 3 weeks; denominators show the number of mice inoculated. The mice inoculated with Sarcoma 37 in one groin were inoculated with Sarcoma DBA 49 in the opposite groin at the same time. The strain C mice with Sarcoma DBA 49 differ significantly from nonimmune hosts.

STRAIN DBA HOSTS		STRAIN C HOSTS	
Secondarily inoculated with sarcoma			
DBA 49	S-37	DBA 49	S-37
18/19		0/14	
6/6	6/6	0/18	18/18

Practically all strain DBA mice took the secondary inoculation. However, in strain C mice, the secondary inoculation of DBA Sarcoma 49 was never successful after the tumor had grown in the tail. Further experiments with two different tumors for secondary inoculation were performed. The primary inoculation in the tail was, again, Sarcoma 49; after the tails had been amputated the mice were inoculated with Sarcoma 49 in one groin and with Sarcoma 37 in the other groin. As before, Sarcoma 49 grew secondarily in strain DBA mice but not in strain C mice. However, both groups of mice grew Sarcoma 37 on secondary inoculation. Do these facts represent some sort of immunology or immunogenetics? I do not know. There are many things about the results which seem to be immunologic in concept and upon which genetics undoubtedly has an influence. Certainly it is easiest to speak of these phenomena when using the terminology of both genetics and immunology.

With this favorable material at our disposal it seemed advisable to see if something could be found out about the nature of the antigen which induces resistance under these circumstances. The early work of Bashford and his co-workers (7) had shown that defibrinated blood could induce resistance. They had also shown that this property was shared in greater or lesser degree by many, if not all, cells and was not a special property of the blood cells. However, the blood cells are one of the easiest to obtain and use and are far better adapted to our purposes than other available cells. It was necessary at first to re-examine some old facts before proceeding to more detailed studies of the factors involved. Accordingly, the blood was separated into serum and cells by centrifugation, and the power of each to induce resistance was tested. The results shown in Table 3 make it quite clear that the power to induce resistance under these circumstances resides in the cells and not in the serum. This is a confirmation of an earlier statement of Bashford *et al.* A further, more precise, examination of these facts appeared necessary as a background for work to be described later, and a more complicated separation of the blood elements was performed. Defibrinated blood from strain DBA mice was layered in a centrifuge tube upon a solution of bovine albumin adjusted to a specific gravity of 1.080 and centrifuged. After centrifugation it was possible to separate four layers, one of red blood cells, a second of bovine albumin, a third of serum, and a fourth, lying at the juncture of the serum and albumin, containing the white cells. None of these layers was entirely clean, but an investigation of the effect of dosage which was conducted at the same time indicated that the small number of cells which contaminated alternate layers was insufficient to produce resistance. An aliquot of defibrinated blood which had been held back during these operations served as a control. The results obtained when the power of these materials to induce resistance in strain C mice were tested are shown in Table 4. The amount of handling required by this procedure resulted in some damage to the cells, and their power to produce resistance was slightly reduced—a common experience in this work; but it is obvious that a highly significant degree of resistance was induced by the blood, and the separated red cells had a potency

TABLE 3

COMPARISON OF THE RESISTANCE OF STRAIN C HOSTS TO INOCULATION OF SARCOMA DBA 49 AFTER A PRIOR INJECTION OF CELLS OR SERUM OF STRAIN DBA BLOOD

Numerators show "takes" at 3 weeks; denominators show total mice inoculated. The difference is highly significant statistically; $P < 0.001$

Cells	Serum
2/13	14/15
1/26	19/27
0/15	13/15
3/54	46/57
5.6 per cent	80.7 per cent

that was similar to that of whole defibrinated blood. The white cells had an intermediate power to induce resistance, and we believe that this was due to dosage. Because nothing further in the work depended upon it, we did not care to investigate this further; but if one recalls the numerical relationship of the blood cells it appears reasonable to explain this in terms of dosage, although other factors are not excluded. The serum, again, had no power to induce resistance, nor did the bovine albumin.

We did not pursue the question of dosage to an ultimate conclusion but did try to determine a much lower dose than that previously used in order to carry out succeeding experiments in a sensitive range and to be sure that the small numbers of residual cells which might not be destroyed or separated by our procedures were insufficient to produce a significant degree of resistance. Starting with the dose previously mentioned, i.e., 0.2 cc., we tried progressively smaller doses of both blood and washed red cells. (We shall speak of equivalent doses hereafter, i.e., the volume of blood from which the materials were derived). It was determined that a dose as small as 0.0031 cc. of washed cells would induce a significant degree of resistance (about 55 per cent takes). This is less than 1 per cent of the dose once thought necessary to produce this phenomenon, but it contains many times the number of cells that were present as residues or contaminants in our other experiments. Finally, a dose of 0.02 cc. of washed red cells was adopted for further work. This dose results in 20–25 per cent takes, a result that is quite different from the 90 per cent takes in controls, and a convenient one to use.

TABLE 4

DEGREES OF RESISTANCE TO SARCOMA DBA 49 INDUCED IN STRAIN C MICE BY THE PRIOR INJECTION OF BOVINE ALBUMIN AND FRACTIONS OF STRAIN DBA BLOOD

Number of "takes" at 3 weeks is shown by the numerator; total number of mice inoculated is shown by the denominator

	IMMUNIZING MATERIAL INJECTED			
Blood	RBC	WBC	Serum	Albumin
5/19	6/17	13/18	15/19	11/15
1/19	1/17	6/19	18/18	12/15
3/19	4/16	6/15	12/16	18/20
9/57	11/50	25/52	45/53	41/50
16 per cent	22 per cent	48 per cent	85 per cent	82 per cent

Next a search was made for methods which would thoroughly disrupt the cells physically without being open to too much suspicion on chemical grounds. It is recognized that one cannot completely separate physics and chemistry in this sense and that anything that might be done to such a delicate system as a red cell could be suspected of producing some chemical change. However, after some trial and error we arrived at methods that appeared to be open to the minimum of such suspicion but which would yield maximum changes in the data. The aggregated results of several experiments with appropriate controls are shown in Table 5. Some of the experiments involved considerable handling of the cells, and the over-all results with intact cells were somewhat higher than might be expected; but the 32 per cent successful grafts observed differs from nonimmune hosts and from the other results at a high level of significance. In one method used to destroy the antigen, the washed red cells were resuspended in cool distilled water, a few seconds were allowed for laking, and sufficient salt was added to bring the tonicity to normal. Laking was quick and efficient. The few cells and large fragments which remained in the suspension were not removed but were injected along with the bulk of the material. The injection of such material into strain C mice did not produce resistance, and the mice were not detectably different from nonimmune animals. Cells exposed to high pitched sound at a frequency of 9 kc. for periods of 90–120 seconds were almost entirely broken up, and after such treatment a suspension of red cells in saline could no longer induce resistance. The power to induce resistance was also destroyed by subjecting a cell suspension to slow freezing and centrifugation. The cell suspension was placed in a centrifuge tube and frozen in the ice compartment of a refrigerator at $-15°$ C. for 1 hour. The frozen material was then placed in a centrifuge at room temperature and spun at 2,000 r.p.m. As the material thawed in the centrifuge, most of the cells were broken up; the number of intact cells that remained was reduced to an inconsequential figure by a second run. (Our experience suggests that the detail of thawing while under centrifugation was a large factor in the efficiency of this method of breaking up the cells). Red cells treated in this manner lost all power to induce re-

TABLE 5

THE DEGREE OF RESISTANCE TO SARCOMA DBA 49 INDUCED IN STRAIN C MICE BY THE PRIOR INJECTION OF STRAIN DBA RED CELLS WHICH HAD UNDERGONE VARIOUS TREATMENTS FOR LAKING

The result in the controls is different from each of the other 3 at a high level of significance; $P < 0.001$

Treatment of cells before injection	No. hosts	No. with tumor	Per cent positive
None (unlaked controls)	204	66	32
Laked by freezing	58	53	91
" " water	59	53	90
" sonically	55	50	91

sistance under the conditions of these experiments. (Complete details of the experiments just described briefly can be found in a recent publication [6]).

What is the nature of the antigen that yields such results as these? We do not think that we know its ultimate nature, but the results reported so far suggest that something critical in the power to produce this type of immunity is linked with the architectural integrity of the cell, its surface, or a major portion thereof. All the methods used are subject to some suspicion on chemical grounds, but other delicate antigens and biological materials have withstood more vigorous applications of the same methods, and it seems unlikely that all three

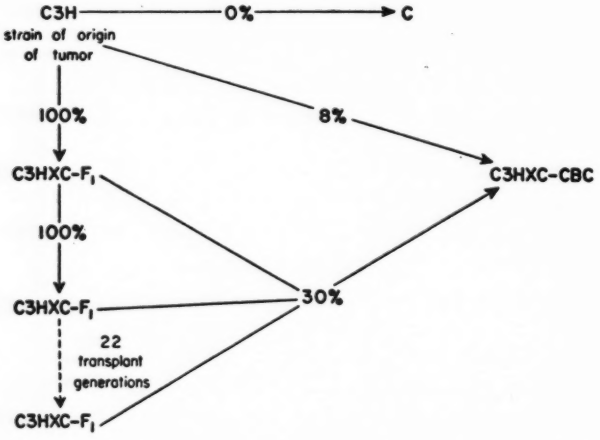


CHART 2.—Summary of the percentages of progressively growing tumors that were observed when the tumor was transplanted between animals of varied genetic constitution. The arrows indicate the donor-recipient relationships. The percentage of the hosts killed by the tumor is shown on the arrows. The differences are all highly significant; $P < 0.001$.

methods would have given rise to the same error. We do not consider the results to be conclusive, but we believe that they suggest an interpretation more readily stated in physical terms than in chemical terms. How much of this could be looked upon as immunology is uncertain. It appears that one may speak of the hosts as being immune or nonimmune and that genetics plays an important part in determining the outcome. The outcome also seems to depend upon the physical state of the antigen. Just what that may mean for the future is not sure.

We have investigated another phenomenon to which the term "immunogenetics" may be applicable. To refresh the memories of those who are not working in this or a related field, we may recall that the general laws in the transplantation of normal tissues and, more particularly, tumors have been known for some time (11). A tumor originating in an inbred mouse can be transplanted to all mice of the same strain and to none of an unrelated strain of mice. It also grows successfully in all F_1 hybrids of which the strain of origin of the tumor furnished one parent and in all susceptible backcross mice derived by mating such an F_1 hybrid to the strain of origin. F_2 animals are derived by mating two F_1 hybrids. In such F_2 hybrids the tumor will grow in 75 per cent, or less—usually much less—depending upon whether one or more so-called "factors" are involved in the transplantation. Resistant backcross animals are derived by mating an F_1 hybrid animal to an animal of the unrelated strain. Such backcross animals are susceptible to the tumor in 50 per cent of cases, or less—usually much less—again depending upon the number of factors involved.

The successful transplantation of a tumor may be said to depend generally upon the genetically determined compatibility of the tumor tissue and the host. We may ask whether the compatibility of the tumor and the host is of an immunologic nature. It has been spoken of frequently in such terms, and it is easy to illustrate the phenomena, if not to explain them, by saying that under these conditions the tumors contain antigens which are compatible or noncompatible with the host and that these antigenic compatibilities are the factors which control the percentage of successful transplants obtained. Keeping these general rules in mind, we pass on to some specific examples. We have worked with a tumor which originated in strain C3H, is transplantable to all animals of that strain, and is not transplantable to animals of strain C. We found by experiment that 8 per cent of resistant backcross animals, produced by mating an F_1 hybrid between strains C3H and C to a strain C animal, succumbed to the tumor. Your attention should be called to the fact that previously I spoke of "takes." The results shown in Chart 2 and thereafter are not based upon "takes," but are based upon the number of animals killed by the tumor. This is not a matter of critical importance in the concepts under discussion but is of some interest otherwise. It was observed further that the tumor was lethal for 8 per cent of resistant backcross hosts only in those cases in which the inoculum came from a stock tumor that had been maintained in strain C3H mice. If, on the other hand, the tumor had grown in F_1 hybrids, either for one generation or up to 22 generations, and was then transplanted into backcross hosts, 30 per cent of the resistant backcross animals were killed by the tumor (4). Is this an immunogenetic phenomenon? Have the immunologic properties of the tumor, which were observed to be based upon genetic constitution at the outset been changed by growing the tumor in an animal that does not have exactly the same genetic constitution? We do not

insist that this is the case, but merely point out that it is easy to speak of the results and to interpret them in such terms. It is possible that we have hit upon a method for repeatedly inducing a change in a tumor which may represent something like what Gorer meant when he spoke of the "immunologic simplification" of tumors (9). In an effort to learn more of the nature of this change we have done a number of other experiments (5). The detailed data of these are too extensive to be presented in their entirety at this time. However, Chart 3 will serve to summarize. This chart shows the results of inoculating four different sub-lines of the same tumor into similar populations of resistant backcross hosts and comparing the outcome to direct transplantation from stock tumors growing in C3H animals. The 8 per cent of lethal tumors that resulted after direct transplantation and which were illustrated in the previous figure are repeated here. The first subline represents the 30 per cent of lethal tumors that resulted from transplantation to backcross hosts after a period of growth in F_1 hybrids. In order to discover whether the change in the tumor tissue was of a relatively permanent or relatively transient type, we maintained a subline of adapted tumors in backcross hosts for 30 generations. The accumulated data from this group of adapted tumors here called the second subline, indicate that these tumors were able to kill 33 per cent of resistant backcross hosts. This is not significantly different from the earlier result of 30 per cent. We wished also to know whether this change in the power of the tumor to grow in the backcross host was dependent upon its maintenance in the relatively foreign host and whether it could be changed back to its original condition by maintaining growth for a number of generations in the strain of origin. Accordingly, from the seventh generation in hybrids the adapted tumor was returned to strain C3H hosts. It was maintained in these hosts for five generations, and at each generation it was tested for its power to kill resistant backcross hosts. This experiment was repeated 3 times. The aggregated results show that 34 per cent of the backcross hosts succumbed to this third subline of the tumor. That is to say, returning the adapted type of tumor tissue to the strain of origin did not reverse the change in transplantability. It was also of interest to find out whether the change in the tumor depended upon its passage through more than one partly foreign host or whether a change occurred during the first generation in F_1 hybrids. Accordingly, a stock tumor was put into F_1 hybrids for one generation. A tumor from this group was selected at random and returned to strain C3H hosts where it was maintained for five generations. The aggregated results with this fourth subline of the tumor show that such a tumor, when transplanted into resistant backcross hosts, has the power to kill 22 per cent. At this point one might indulge in a statistical debate as to whether all these sublines are alike. Statistically it is obvious that all four sublines are different from the stock tumor which kills only 8 per cent of backcross hosts. However, it is not certain that the fourth subline which kills

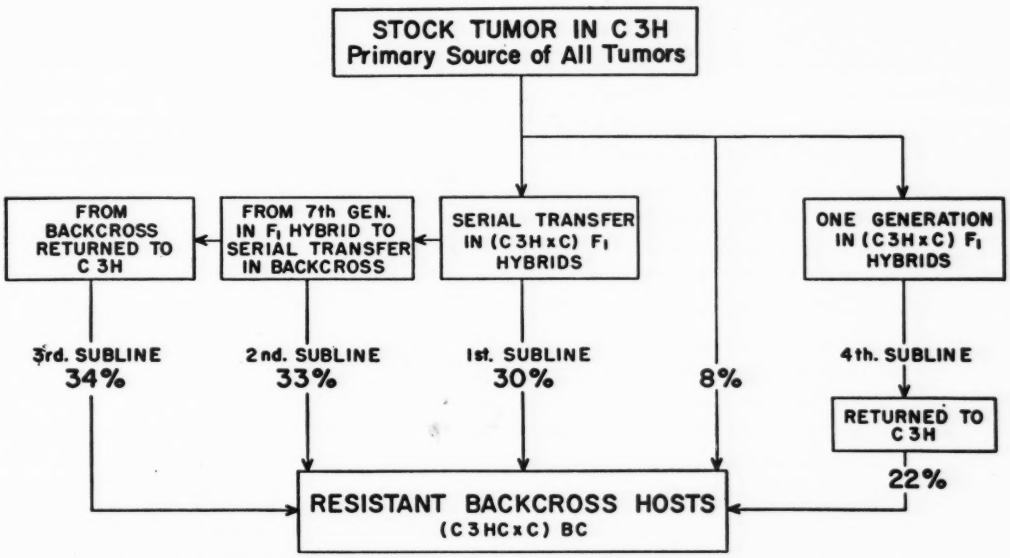


CHART 3.—Showing the percentage of tumors which grew progressively in backcross hosts after varied transplantation histories for the tumor. The position of the boxes and the arrows indicate the genealogies of the tumors and not that of the hosts. The four sublines of the tumor referred to in the text are shown. Note that for all tumors the primary source and the ultimate host are the same in all cases. Over 1,000 hosts and nearly 300 tumors are represented. See (5) for statistical discussion.

22 per cent is the same as the other three sublines which killed 30–34 per cent. It is possible that the result of 22 per cent represents a first and major change in the ability of the tumor to grow in backcross hosts and that the difference between this percentage and the others represents more subtle changes which occur gradually during long periods of transplantation. We do not know the answer to this question, but we consider that such an interpretation could be adopted, for the present at least. It is clear, however, that the change which takes place in the tumor is of a relatively permanent kind.

We do not know the exact nature of this change in the tumor, nor do we know a great deal about the specificity of the change, although extensive data are being accumulated which will bear on this point. I wish, however, to call your attention to the data in Table 6 in order to emphasize the point that we are not dealing with a general loss in specificity of the tumor. The data shown make it clear that whatever the nature of the change, it is not simply a generalized loss in specificity such as has sometimes been observed. At the present time we know that the tumor is still in its original condition with respect to transplantability as far as we can determine, although it is in the 115th transplant generation; it will grow in 100 per cent of C3H mice and in 100 per cent of C3H × C F_1 hybrids, regardless of the donor that contributes the tissue. In the case of resistant backcross hosts it grows in the variable percentages shown, depending upon whether the donor of the tumor tissue was a strain C3H or an F_1 hybrid. In second backcross animals we again got variable results depending upon the donor of the tumor tissue. These second backcross animals were not derived by specially selecting the first backcross animals with regard to their susceptibility to the tumor, and therefore, we do not think that these animals represent the highest degree of genetic control that might have been used. The thought in mind at the time these animals were produced was to see if the constitution of the animal with regard to the average proportion of chromatin material from a given strain might have a striking effect upon the results. We do not consider that very much can be made of the data from second backcross hosts, and the statistical significance is not high; but the contention that the tumor has not undergone a general loss of specificity is strongly supported. However, the important point is that when strain C mice were inoculated with this tumor from the three available sources the results made it clear that, although the tumor has acquired some enhanced power to grow in an animal which is partially derived from strain C, it has not become nonspecific and still has very limited, if any, power to grow in a strain C mouse. Thus, the changes we have shown appear to be fractional and do not reflect a complete loss of specificity. Whether all this is immunology or not, we do not know. It is, of course, obvious that much of it is genetics. Many of the working hypotheses under which these experiments have been carried out have been immunologic in concept, and we think that the hybrid word "immunogenetics" may be, at least for the moment, properly applied to much of this work.

TABLE 6

ACCUMULATED RESULTS SHOWING THE GENERAL TRANSPLANTABILITY OF MAMMARY ADENOCARCINOMA C3HBA AS INFLUENCED BY THE GENETIC CONSTITUTION OF DONOR AND RECIPIENT

The data represent progressively growing tumors. There are both 1st and 2d backcross to strain C among the recipients and 1st backcross among the donors as indicated

Recipient of tumor	Donor of tumor	Total mice inoc.	Total tumors	Per cent positive
C3H	Any	1,000 plus		100
C3H×C F_1	Any	1,000 plus		100
(C3HC×C)BC	C3H	281	22	7.8
(C3HC×C)BC	F_1	175	42	24.0
(C3HC×C)BC_2	C3H	310	5	1.6
(C3HC×C)BC_2	F_1	89	2	2.2
(C3HC×C)BC_2	Backcross	178	12	6.7
C	C3H	478	0	0
C	F_1	115	0	0
C	Backcross	265	2	0.8

REFERENCES

1. ANDERVONT, H. B. Studies on Immunity Induced by Mouse Sarcoma 180. Pub. Health Rep., **47**:1859–77, 1932.
2. ———. Use of Pure Strain Animals in Studies on Natural Resistance to Transplantable Tumors. *Ibid.*, **52**:1885–95, 1937.
3. BARRETT, M. K. The Influence of Genetic Constitution upon the Induction of Resistance to Transplantable Mouse Tumors. J. Nat. Cancer Inst., **1**:387–93, 1940.
4. BARRETT, M. K., and DERINGER, M. K. An Induced Adaptation in a Transplantable Tumor of Mice. J. Nat. Cancer Inst., **11**:51–59, 1950.
5. ———. Induced Adaptation in a Tumor: Permanence of the Change. *Ibid.*, **12**:1011–17, 1952.
6. BARRETT, M. K.; HANSEN, W. H.; and SPILMAN, B. F. The Nature of the Antigen in Induced Resistance to Tumors. Cancer Research, **11**:930–36, 1951.
7. BASHFORD, E.; MURRAY, J. A.; and CRAMER, W. The Natural and Induced Resistance of Mice to the Growth of Cancer. Third Sc. Rep., Imp. Cancer Res. Fund, 315–40, 1908.
8. CUMLEY, R. W.; IRWIN, M. R.; and COLE, L. J. Genetic Effects of Serum Proteins. Proc. Nat. Acad. Sc., **27**:565–70, 1941.
9. GORER, P. A. The Significance of Studies with Transplanted Tumors. Brit. J. Cancer, **2**:103–7, 1948.
10. LANDSTEINER, K. The Specificity of Serological Reactions. Rev. Ed. Cambridge: Harvard Univ. Press, 1946.
11. LITTLE, C. C. The Genetics of Tumor Transplantation. In Biology of the Laboratory Mouse, pp. 279–309, 1941.
12. NUTTALL, G. H. F. Blood Immunity and Blood Relationship. Cambridge: at the University Press. 1904.
13. WOGLOM, W. H. Immunity to Transplantable Tumours. Cancer Rev., **4**:129–214, 1929.

Paper No. 2 in the Symposium on Immunogenetics and Carcinogenesis

The Immunogenetics of Tumor Transplantation*

GEORGE D. SNELL

(Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine)

The current program of transplantation research at the Jackson Laboratory is based on two approaches: one immunological in conception and, probably, in fact; the other genetic. As yet the only certain connection between the two is their common dependence on transplantable tumors. Some preliminary evidence, however, points to other interesting relationships.

This has been a joint project. Much of the work has been done by Dr. Kaliss and Dr. Borges. At an earlier date, Dr. Cloudman also participated. Drs. Molomut and Spain have collaborated in certain aspects of the undertaking. I shall also review some results, possibly related to ours, obtained at other laboratories.

The genetic investigations have been concerned with the detailed analysis of the genes determining susceptibility and resistance to transplants. I will not describe the methods used, since these are quite technical, but instead will give a brief summary of conclusions.

One genetic locus has turned out to be particularly important in determining susceptibility and resistance to transplants. This locus has been termed histocompatibility-2 or *H-2* (25). It is a "strong" locus, that is to say, a difference at this locus between tumor and host will prevent the progressive growth of all but a very few of the most virulent transplantable tumors. The existence of six alleles or alternative forms has been established. These are shown in Table 1, together with their distribution in fifteen inbred strains. It seems likely that additional alleles will be found as more strains are tested.

An interesting characteristic of the *H-2* locus is the fact that the allele $H\text{-}2^{dk}$, found in strain A, has two components, d and k, which are present separately in certain other strains (22). Thus, strains BALB/c and DBA/2 have the d component, while strains C57BR/a, C57BR/cd, AKR, CBA, C3H, and ST have the k component. All F_1 hybrid mice of the genotype $H\text{-}2^{d}/H\text{-}2^{k}$ will grow strain A ($H\text{-}2^{dk}/H\text{-}2^{dk}$) tumor 15091a regardless of which of the d and k strains is involved in the cross. Mice from any other type of cross are resistant (Snell, unpublished data).

Strain C57BL-x (Table 1) is interesting in that it arose by mutation from strain C57BL, allele $H\text{-}2^{b}$ changing to allele $H\text{-}2^{d}$ ([3] and Snell, unpublished data).

A second aspect of immunogenetics currently under investigation at the Jackson Laboratory is the enhancing effect. This effect was first noted by

TABLE 1

ALLELES AT THE HISTOCOMPATIBILITY-2 LOCUS AND THEIR DISTRIBUTION IN DIFFERENT INBRED STRAINS

$H\text{-}2^{dk}$	$H\text{-}2^{d}$	$H\text{-}2^{k}$	$H\text{-}2^{b}$	$H\text{-}2^{p}$	$H\text{-}2^{a}$
A	BALB/c	C57BR/a	C57BL	P	DBA/1
	DBA/2	C57BR/cd	C57L		
	C57BL-x	AKR	LP		
		CBA	129		
		C3H			
		ST			

Flexner and Jobling (11), who found that pretreatment of rats with heat-killed tumor tissue enhanced the growth of subsequently inoculated living tumor. More detailed studies of the phenomenon were carried out by Casey (4–6) with rabbits and mice. Casey prepared tumor tissue for pretreatment of host animals by storing the tissue at freezing temperatures until it no longer caused tumors when inoculated. Snell *et al.* (23), using mouse tumors transplanted in a foreign, and hence resistant, inbred strain, found that pretreatment with lyophilized tumor tissue in certain combinations of host and donor produced the effect to a striking degree, and Kaliss and Snell (15) demonstrated its occurrence following pretreatment with lyophilized normal tissue. In appropriate tumor-host combinations, in which nearly 100 per cent of untreated mice resist the growth of transplants,

* The studies at the R. B. Jackson Memorial Laboratory on which this paper is based have been assisted by a grant from the National Cancer Institute and by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

80–100 per cent of animals preinjected with lyophilized tumor succumb.

The usual dose for producing the enhancing effect is 30–50 mg., given in one or more injections. An important step forward was taken when Kaliss and Newton (14) demonstrated that doses of 0.05 mg. would produce inhibition instead of enhancement. For this purpose Dr. Kaliss selected a tumor-host combination in which approximately 50 per cent of the control animals succumbed. This made it easy to determine a shift either up or down in susceptibility. With tumor E 0771 (C57BL origin) in C3H mice, the percentages of mice dying were: 0.05 mg., 21 per cent; 0.5 mg., 68; 5.0 mg., 74; 50.0 mg., 94; and control, 41 per cent. This result has now been repeated and confirmed with the same and other tumor-host combinations (16). In these more recent experiments, doses of both 0.05 and 0.005 mg. have been shown to produce inhibition.

The usual interval between the last injection of frozen-dried tissue and the inoculation of fresh tumor is 1–3 weeks. However, the effect persists longer than this. Of ten C57BR/a mice given 30 mg. of lyophilized 15091a and inoculated 51 days later, nine succumbed. All controls exhibited negative results (Snell, unpublished data). Casey (7) reported the persistence of the effect for 7 months in the rabbit.

Kaliss and Borges (12) ran parallel tests of trypan blue and lyophilized tumor tissue. Mice receiving the latter showed the usual enhancement of tumor growth, those receiving the former showed none.

Casey (4) and Kaliss and Borges (12) both found the effect to be species-specific. Rabbit tumor preparation and lyophilized normal rat, hamster, guinea pig, and rabbit tissues injected in mice failed to produce the enhancing effect on mouse tumors.

Casey (5, 6) reported greater enhancement when the same mouse tumor was used for pretreatment and for inoculation than when different mouse tumors were used.

We are particularly interested at the present time in this question of specificity. The results to date are presented in Table 2. I am indebted to Dr. Kaliss for some of the data included. The tumor used was 15091a, strain A origin. The hosts were C57BL/6Ks and C57BR/a, which had been pretreated with lyophilized liver or kidney from different inbred strains. It will be noted, first, that strain A tissues, homologous with the test tumor, gave a maximum effect. At the other extreme, C57BR/a tissue in C57BR/a mice, and P tissue in both C57BL and C57BR/a mice gave a minimum effect. Tissues from other sources gave intermediate values, except that BALB/c and DBA/2 tissues in C57BR/a mice are equally as effective as A tissues. These intermediate values are compatible with the assumption that enhancement depends on pretreatment with the histocompatibility genes (or their products) which are present in the test tumor but absent from and hence foreign to the host. Further tests are in progress.

TABLE 2

PER CENT MICE DYING FROM TRANSPLANTS OF 15091a (dk*) FOLLOWING PRETREATMENT WITH LYOPHILIZED NORMAL TISSUE FROM DIFFERENT INBRED STRAINS

	Normal tissue from						
Host strain	A *dk	BALB/c DBA/2 d	CBA ST k	C57BL b	P p	C57BR/a k*	Control
	(per cent)						
C57BL/6Ks b*	50 (16)	6 (38)	40 (20)		0 (22)		0 (38)
C57BR/a k*	44 (68)	45 (69)	29 (68)	31 (19)	11 (19)	12 (49)	4 (49)

* *H-2* allele, indicated by superscript or "component" symbol.

Number of mice given in parentheses.

In an extremely interesting development, Kaliss and Molomut (13) have shown that something equivalent to a "passive transfer" of the enhancing effect can occur. Mice and rabbits were given the standard injections of lyophilized 15091a, and their sera were injected by the intraperitoneal route into other mice. There was greater growth of 15091a in the injected mice than in controls. The interpretation of these results is still obscure.

Kaliss and Spain (16) have shown that there is some increased growth of grafts of embryonic tissue and of spleen in hosts pretreated with lyophilized tissue, but the effect is slight compared to that on tumor transplants.

I now turn to some studies at other laboratories which present interesting parallels to the enhancing effect.

It is well known that growth and regression of a tumor graft in a foreign strain evokes an immunity such that a second graft usually shows no growth at all. Lewis and Lichtenstein (17, 18) tested the effect of repeated grafts, up to a dozen or more, from one inbred strain into another. The result of this treatment was that the tumors eventually began to grow in the mice. Here, as in our experiments with the enhancing effect, we find a small dose (one graft) producing immunity, a large dose (multiple grafts), susceptibility.

Felton and co-workers (8–10), studying the reaction of mice to pneumococcus polysaccharides, types I, II, and III, found a similar dosage phenomenon. An injection of 0.0005 mg. immunized mice against the virulent pneumococcus. On the other hand, mice receiving 0.5 mg. showed what Felton called "immunological paralysis." In these mice the immunizing dose of 0.0005 mg. no longer evoked antibody formation, nor did it protect against the living pneumococcus. The effect was species- and type-specific and persisted for 15–18 months after one "paralyzing" injection. The polysaccharide could still be demonstrated in the tissues 1 year after the injection.

The fourth series of studies is concerned with fraternal twins in cattle. Owen and co-workers (20, 21) made the interesting discovery that cattle twins, whether one-egg or two-egg, usually have identical blood groups. Without going into the details of the evidence, we may say that red cell precursors from one calf pass through anastomoses in the placentas and become established in the hemapoietic tissues of the twin. In certain cases it was demonstrated that calves had cells of two distinct antigenic types, corresponding to the genotypes of the two members of the twin pair. Subsequently, Anderson *et al.* (1) found that skin can be grafted successfully between dizygotic calf twins. Ten twin pairs were tested. The animals were, as expected, resistant to grafts from any animal but the twin, the graft in these cases breaking down rapidly and completely. In reciprocal grafts between the twins, grafts in six cases were completely successful, behaving essentially like autografts. In two additional cases the grafts were successful, but an inflammatory reaction gave evidence of some incompatibility. In two cases the grafts broke down, but much more gradually than typical homografts.

Here again we find that hosts that have received massive doses of tissue (in this case, blood) from a donor lose their normal resistance to grafts from the donor. The effect is specific; grafts from other donors are still resisted. An interesting question is whether here, as in the enhancing effect and Felton's "immunological paralysis," small doses reverse the phenomenon and produce immunity. It is impossible to compare the effects of small doses of blood reaching a calf under conditions strictly comparable to those obtaining in twins. However, the injection of blood has been used in mice to immunize against tumor transplants (2) and in rabbits to immunize against skin grafts (19). In the latter case it is the white blood cells that seem to be the effective immunizing agent.

TABLE 3

COMPARISON OF ENHANCING EFFECT WITH OTHER POSSIBLY RELATED PHENOMENA

	Authors	Pretreatment with	Nature of response	Small dose inhibits	Effect specific	Effect persists
Enhancing effect	Snell and Kaliss, Casey	Lyophilized or otherwise killed tissue	Enhanced susceptibility to transplants	Yes	Yes	Yes
Repeated inoculation effect	Lewis and Lichtenstein	Fresh tumor	Enhanced susceptibility to tumor transplants	Yes	?	?
"Immunological paralysis"	Felton	Killed bacteria or bacterial polysaccharides	Enhanced susceptibility to bacteria	Yes	Yes	Yes
Skin graft susceptibility in twin cattle	Anderson *et al.*	Red cells or red cell precursors	Enhanced susceptibility to skin grafts	Yes?	Yes	?

In Table 3 the different phenomena that we have been discussing are compared. As far as the evidence goes (there are several gaps), all these cases show: (*a*) the production of susceptibility to foreign cells by pretreatment with large doses of killed cells or cell products; (*b*) the production of resistance with small doses; (*c*) specificity—pretreatment for any or for the maximum effect must be with a tissue or extract genetically related to

the challenging cells; (*d*) persistence—the effect does not wear off immediately after the treatment ends. Because of these similarities, it would seem justifiable to postulate that the enhancing effect produced by lyophilized tissue, the enhancing effect produced by repeated tumor grafts, Felton's "immunological paralysis," and the graft susceptibility of dizygotic twin calves are fundamentally the same phenomenon.

ADDENDUM

Since this paper was presented, another publication (Kearns, J. E., Jr., and Reid, S. E. Successful Homotransplantation of Skin from Parents to Son. Plastic and Reconstructive Surgery, **4**: 502–7, 1949), describing results possibly related to the enhancing effect, has come to my attention. The authors describe the case of a 9-year-old boy who came under their care 8 months after sustaining a severe burn of the right leg. The burn was infected and still unhealed. Autotransplants of skin had been unsuccessful. The boy was of the same blood group as both parents and "had previously received repeated transfusions from each without exhibiting evidence of sensitization." Over a period of several weeks skin grafts were made to the damaged areas from both the mother and father. "These grafts persisted, and subsequently the areas between them were covered by epithelium growing from their margins and the adjacent areas of the patient's skin."

Too much weight cannot be attached to a single case, but it is interesting to inquire whether this unique case of successful homotransplantation of skin may not have been due to the prior blood transfusions, and, hence, still another parallel of the enhanced growth of tumor and normal tissue transplants produced by prior injections of lyophilized tissue.

REFERENCES

1. Anderson, D.; Billingham, R. E.; Lampkin, G. H.; and Medawar, P. B. The Use of Skin Grafting To Distinguish between Monozygotic and Dizygotic Twins in Cattle. Heredity, **5**:379–97, 1951.
2. Barrett, M. K. The Influence of Genetic Constitution upon the Induction of Resistance to Transplantable Mouse Tumors. J. Nat. Cancer Inst., **1**:387–93, 1940.
3. Borges, P. R. F., and Kvedar, B. J. A Mutation Producing Resistance to Several Transplantable Neoplasms in the C57 Black Strain of Mice. Cancer Research, **12**:19–24, 1952.
4. Casey, A. E. A Species Limitation of an Enhancing Material Derived from a Mammalian Tumor. Proc. Soc. Exper. Biol. & Med., **30**:674–77, 1933a.
5. ———. Failure of a Mouse Carcinoma Material To Enhance a Mouse Sarcoma. *Ibid.*, 1025–26, 1933b.
6. ———. Specificity of Enhancing Materials for Mammalian Tumors. *Ibid.*, **31**:663–65, 1934a.
7. ———. A Persistent Hypersusceptibility Induced in Rabbits with an Homologus Tumor Material. *Ibid.*, 666–67, 1934b.
8. Felton, L. D. The Significance of Antigen in Animal Tissues. J. Immunol., **61**:107–17, 1949.
9. Felton, L. D., and Attinger, B. Pneumococcus Polysaccharide as a Paralyzing Agent on the Mechanism of Immunity in White Mice. J. Bact., **43**:94–95, 1942.
10. Felton, L. D.; Prescott, B.; Kauffmann, G.; and Attinger, B. Studies on the Immunizing Substances in Pneumococci. XIV. The Distribution of Specific Polysaccharide in Mouse Tissues after Injection of a "Paralyzing" Dose. Fed. Proc., **6**:427, 1947.
11. Flexner, S., and Jobling, J. W. On the Promoting Influence of Heated Tumor Emulsions on Tumor Growth. Proc. Soc. Exper. Biol. & Med., **4**:156–57, 1907.
12. Kaliss, N., and Borges, P. R. F. The Effect of the Injection of Non-Mouse Substances on the Growth of Tumor Homoiotransplants in Mice. Cancer Research, **11**:260, 1951.
13. Kaliss, N., and Molomut, N. The Effect of Prior Injections of Tissue Antiserums on the Survival of Cancer Homoiografts in Mice. Cancer Research, **12**:110–12, 1952.
14. Kaliss, N., and Newton, O. The Effect of Injection Dosage Level of Lyophilized Mouse Tissue on the Subsequent Growth of a Tumor Homoiotransplant. Anat. Rec., **105**:535, 1949.
15. Kaliss, N., and Snell, G. D. The Effects of Injections of Lyophilized Normal and Neoplastic Mouse Tissues on the Growth of Tumor Homoiotransplants in Mice. Cancer Research, **11**:122–26, 1951.
16. Kaliss, N., and Spain, D. M. The Effect of Prior Injections of Lyophilized Mouse Tissues on the Survival of Normal Tissue Homoiografts in Mice. Cancer Research, **12**:272–73, 1952.
17. Lewis, M. R., and Lichtenstein, E. G. Breaking Down the Resistance of Albino Mice to the Transplantation of Tumors Induced by 1:2:5:6-Dibenzanthracene in a Different Strain of Albino Mice. Am. J. Cancer, **27**:246–56, 1936a.
18. ———. Further Studies on the Breaking Down of Resistance of Mice of One Strain to the Transplantation of Tumors from Mice of Another Strain. *Ibid.*, **28**:746–51, 1936b.
19. Medawar, P. B. Immunity to Homologous Grafted Skin. II. The Relation between Antigens of Blood and Skin. Brit. J. Exper. Path., **27**:15–24, 1946.
20. Owen, R. D. Immunogenetic Consequences of Vascular Anastomoses between Bovine Twins. Science, **102**:400–401, 1945.
21. Owen, R. D.; Davis, H. P.; and Morgan, R. F. Quintuplet Calves and Erythrocyte Mosaicism. J. Hered., **37**: 290–97, 1946.
22. Snell, G. D. A Fifth Allele at the Histocompatability-2 Locus of the Mouse as Determined by Tumor Transplantation. J. Nat. Cancer Inst., **11**:1299–1305, 1951.
23. Snell, G. D.; Cloudman, A. M.; Failor, E.; and Douglass, P. Inhibition and Stimulation of Tumor Homoiotransplants by Prior Injections of Lyophilized Tumor Tissue. J. Nat. Cancer Inst., **6**:303–16, 1946.
24. Snell, G. D.; Cloudman, A. M.; and Woodworth, E. Tumor Immunity in Mice Induced with Lyophilized Tissue, as Influenced by Tumor Strain, Host Strain, Source of Tissue, and Dosage. Cancer Research, **8**:429–37, 1948.
25. Snell, G. D., and Higgins, G. F. Alleles at the Histocompatibility-2 Locus in the Mouse as Determined by Tumor Transplantation. Genetics, **36**:306–10, 1951.

Paper No. 3 in the Symposium on Immunogenetics and Carcinogenesis

In Vivo Combinations between Carcinogens and Tissue Constituents and Their Possible Role in Carcinogenesis*

ELIZABETH C. MILLER AND JAMES A. MILLER

(McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison 6, Wis.)

Approximately 20 years have elapsed since the first pure chemical carcinogens were discovered. Yet today we still do not know the exact nature of *any* of the steps in the carcinogenic process which can be initiated by such agents. The present paper is concerned with some recent biochemical approaches to this problem, particularly with the combinations that certain chemical carcinogens or their derivatives form *in vivo* with tissue constituents. These reactions appear to involve the proteins of the cells, and it seems to us that such binding reactions, through altering the characteristics of specific proteins, might play an important part in the induction of tumors. Thus, we (13, 15, 20, 26) and others (25, 29) have visualized carcinogenesis as a resultant of the modification or deletion of certain cellular proteins (enzymes). Through such a process a new race of cells would be formed which differed from the parent cells in protein makeup and which possessed a new balance beween their enzymatic reactions. If these cells had the requisite systems for growth but lacked the enzymatic factors which could limit that growth, they would have the properties of tumor cells. Such alterations in protein makeup might also bring about different immunological properties, but as yet this aspect of the problem has not been studied.

Although there were few data to substantiate the idea until recently, several investigators have suggested that chemical carcinogens might act through *in vivo* combination with cellular constituents. Thus, in 1938 Fieser (5) postulated that the polycyclic hydrocarbons might be converted *in vivo* to derivatives capable of conjugation with tissue constituents. Later he suggested that the polycyclic hydrocarbons, without prior substitution, might react with the –S–S– bridges of proteins (6). Probably the first experimental evidence for the *in vivo* combination of a hydrocarbon with tissue components was presented by Doniach, Mottram, and Weigert (4) in 1943. These authors, working with 3,4-benzpyrene, implicated tissue proteins in the reaction, but much more conclusive evidence for the formation of protein-bound derivatives of the polycyclic hydrocarbons in treated tissues has been obtained recently by Miller (12) and Heidelberger and his associates (9, 39). The idea that carcinogens might combine with tissue proteins or nucleoproteins has also been incorporated by Hendry *et al.* (10) into their polymerization theory of carcinogenesis. On the other hand, Boyland (1), ignoring the published data on the formation *in vivo* of protein-carcinogen compounds (9, 12, 13, 15), has speculated that carcinogens produce their effects through a direct action on chromosome nucleic acids and has even tabulated the possible types of *in vivo* action of a number of carcinogens on desoxypentosenucleic acid. In view of the increasing array of evidence with various classes of carcinogens for the occurrence of protein-carcinogen combinations *in vivo* (see below) and the inability, up to the present, to demonstrate *in vivo* combinations between chemical carcinogens and nucleic acids, this theory appears to have little experimental basis.

The major share of the experimental work on *in vivo* carcinogen-protein combinations has been carried out with the aminoazo dyes which induce liver tumors when fed to rats (13, 15, 21). When

* The work from the authors' laboratory has been supported in part by research grants from the National Cancer Institute of the National Institutes of Health, Public Health Service, the American Cancer Society upon recommendation by the National Research Council, the Alexander and Margaret Stewart Trust Fund, and the Jane Coffin Childs Fund for Medical Research.

4-dimethylaminoazobenzene, which might be considered the parent compound of this series, is fed, the major share of the dye is metabolized via reductive cleavage of the azo linkage, hydroxylation of the ring in the 4′-position, and oxidative removal of the N-methyl groups. However, a small percentage of the dye is metabolized to a derivative which eventually becomes chemically bound to certain of the liver proteins. This product can be demonstrated by immersing formalin-fixed liver slices in a strong acid, since slices from dye-fed rats turn pink under these conditions, while those from rats not fed dye are buff-colored. On transfer to alkaline solutions the pink slices from the dye-fed rats turn slightly yellow, while the normal ones are unchanged. This reversible change from pink to yellow on transfer from acidic to basic media is characteristic of these aminoazo dyes. If the liver is homogenized, the proteins precipitated,

TABLE 1

THE NUCLEIC ACID AND PROTEIN-BOUND DYE CONTENTS OF LIVER PROTEIN BEFORE AND AFTER TREATMENT WITH HOT TRICHLOROACETIC ACID

	Weight (mg.)	Total nucleic acids (mg.)	Bound dye E*
Cold TCA† precipitate	100.0	7.5	0.156
Same, after hot TCA extraction (32)	84.6	0.3	0.167

* E = log I_0/I, measured at 520 mμ under standard conditions (13), for the entire sample.

† TCA = trichloroacetic acid.

and the protein precipitate washed and exhaustively extracted, the resulting protein preparation still has the same color properties as the slice. In fact, the only means by which the dye can be removed is by hydrolysis of the protein with either proteolytic enzymes or strong alkali. These procedures release small amounts of 4-aminoazobenzene and 4-monomethylaminoazobenzene, but the major share has very polar properties and appears to bear new substituents on the amino group. In other respects the polar dye appears to be very similar in structure to the dye administered.

Since protein preparations such as these are a mixture of proteins and nucleoproteins, the dye might be combined with either the protein or nucleic acid moieties or both. However, only the protein fraction appears to be involved directly (Table 1). Thus, in this instance the protein-nucleoprotein mixture, precipitated and extracted according to our usual procedure, contained 7.5 per cent of nucleic acids, while the proteins from an equal aliquot, which was treated similarly but was also extracted with hot 5 per cent trichloroacetic acid according to Schneider's method (32), contained only 0.4 per cent of nucleic acids. The latter precipitate also contained 10 per cent less protein than the first preparation; yet it still contained all the bound dye originally present. Of course, the finding that the dyes are not bound to the nucleic acids directly does not exclude the possibility that they may be bound to the protein moiety of nucleoproteins.

Although we do not know the structure of the protein-bound dyes, we do have some data on the possible reactions involved in the binding. Thus, if the polar dye released by hydrolysis of the bound dye is reduced at the azo linkage, one can distill off a volatile amine which, when 4-dimethylaminoazobenzene is the dye fed, has been identified as aniline. The other fragment, derived from the diamine portion of the dye, is highly polar (15), so that the linkage to protein apparently occurs either through this ring or through a derivative of the N-methyl groups. We feel that the latter site is the one involved. Thus, only dyes with N-methyl groups become bound to the protein to any large extent, and, further, appreciable binding takes place only with those N-methyl dyes in which the methyl groups are readily metabolized. For instance, little binding occurs in the case of the N-benzylmethyl or N-β-hydroxyethylmethyl derivatives, and in these cases the methyl groups are metabolized very slowly (18). *In vitro* studies showed that the N-demethylation reaction is an oxidative process in which formaldehyde and the demethylated dye are the end-products and in which a N-hydroxymethyl derivative is the most likely intermediate (Chart 1) (24). Although 3-methyl-4-monomethylaminoazobenzene was used in these studies, a comparable reaction apparently occurs in the case of other N-methyl dyes. Evidence for this reaction *in vivo* has been obtained by the use of N-methyl–C^{14}-labeled dyes (11, 16). When these dyes are fed to rats, C^{14} is found in the serine of the liver and body proteins and is found exclusively in the β-carbon of this amino acid. This specific localization has been amply demonstrated by others to be the characteristic mode of incorporation of one-carbon compounds such as formaldehyde and formate.

Hence, N-hydroxymethyl or closely allied derivatives appear to be major metabolites of the N-methyl dyes. Probably the major share quickly decomposes into the lower amine and formaldehyde, but some of this derivative might also react with active hydrogens of amino acids in the liver proteins (or with amino acids prior to their incorporation into proteins) to yield dye-protein compounds of the types shown in Chart 2. This sug-

gested reaction mechanism is an application of the Mannich base reaction with proteins which has been studied extensively by Fraenkel-Conrat and Olcott (7). If this reaction occurred with amino acids such as tyrosine or histidine, –N–C–C– bonds would be formed; according to Fraenkel-Conrat and Olcott, this linkage is alkali-stable, and the dyes obtained following alkaline hydrolysis would be expected to carry a polar amino acid residue. On the other hand, if the reaction occurred with an indole, guanidyl, or amide group, the resulting –N–C–N– bond would be expected to be alkali-labile. In this case demethylated dye would be liberated on hydrolysis. Actually, on hydrolysis of the liver proteins from dye-fed rats we do obtain both polar dyes, which constitute about 90 per cent of the total bound dye, and nonpolar dyes, which are a mixture of 4-monomethylaminoazobenzene and 4-aminoazobenzene. Thus, both of the type reactions illustrated may take place, although other reaction mechanisms are certainly not excluded.

So far, attempts to produce protein-bound dye *in vitro* by enzymatic means have been unsuccessful. However, if one warms a suspension of denatured normal liver protein in a solution of 4-aminoazobenzene or 4-monomethylaminoazobenzene and formaldehyde, appreciable amounts of the dye are bound to the protein. Similar binding takes place with denatured protein from azo dye-induced tumors or with crystalline beef serum albumin. Presumably this binding also involves a N-hydroxymethyl intermediate formed by the addition of formaldehyde to the amino group of the dye and subsequent reactions similar to those depicted in Chart 2. Like the product formed *in vivo*, these artificial protein-bound dyes yield both polar and nonpolar dyes on hydrolysis. While the

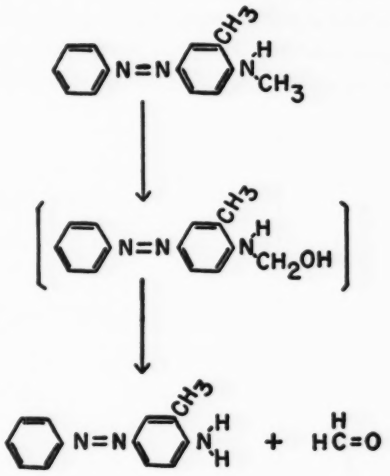


CHART 1.—Reactions involved in the N-demethylation of 3-methyl-4-monomethylaminoazobenzene by rat liver homogenates.

Mannich base reactions are highly suggestive, to know whether or not they really occur we need to determine the nature of the substituents on the polar dyes formed *in vivo*. Recently, we have made some progress in the purification of the polar dyes. By chromatography on Florisil the polar dye has

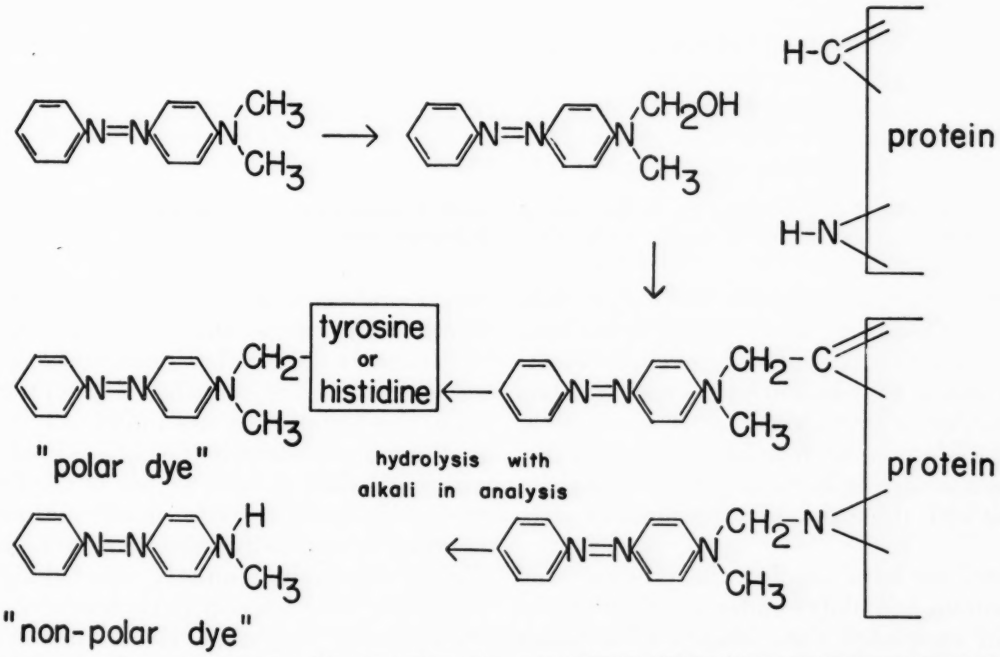


CHART 2.—A possible mechanism for the formation of protein-bound derivatives of methylated aminoazo dyes *in vivo*. (Reprinted from *Advances in Cancer Research*, Vol. 1, with the permission of the publishers.)

been divided into one major and two minor components,[1] and it is interesting to speculate that these dyes might differ only in the nature of the attached amino acid residue.

But what evidence do we have that these protein-bound dyes are actually involved in the genesis of tumors by the azo dyes? At the present time so little is known about the fundamental nature of the carcinogenic process that no tests are available to enable us to identify with precision successive stages in the induction of tumors. The only certain indicator that the process has occurred or has been in progress in a given tissue is the eventual emergence of a tumor. Consequently, the task of determining whether a given reaction is involved in carcinogenesis seems to rest on correlations between events occurring in the target tissue early in the process with the ultimate appearance of gross tumors.

Therefore, we have studied the presence and levels of protein-bound dye under a variety of conditions and correlated these results with tumor susceptibility (13, 15). Thus, of the eight species tested, rats are very susceptible to the carcinogenic activity of 4-dimethylaminoazobenzene, while mice develop liver tumors much more slowly and in a smaller percentage of the animals. Six other species have not developed liver tumors even when the animals were fed the dye at high levels for long periods of time. Similarly, when these animals are fed dye, the livers of rats contain relatively high levels of bound dye, mouse liver contains but low levels, and no bound dye can be detected in the livers of the species that do not develop tumors.

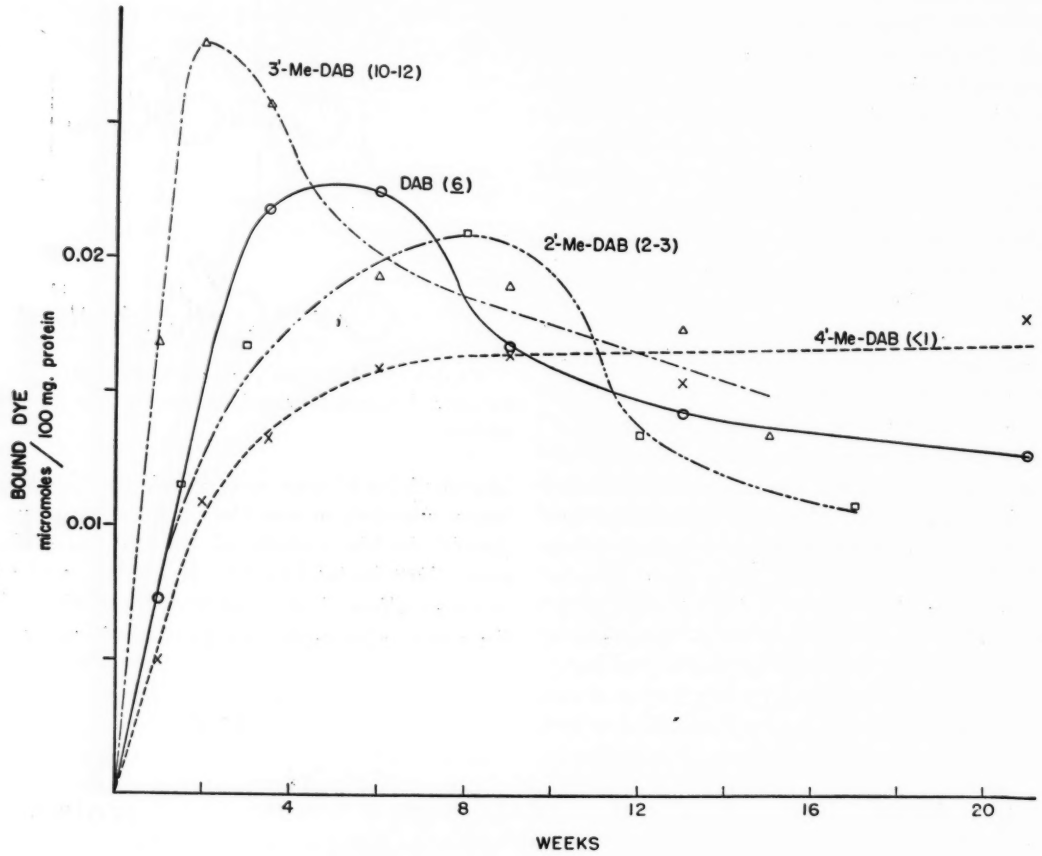


CHART 3.—The levels of protein-bound dye in the livers of rats fed 4-dimethylaminoazobenzene or certain of its ring-methyl derivatives (15). (Reprinted from *Cancer Research*, 9:336–43, 1949.)

Second, 4-dimethylaminoazobenzene is specifically a liver carcinogen when it is fed to rats. Likewise, the bound dyes are found only in the liver and to a small extent in the plasma proteins, which are synthesized at least in part in the liver. Third, the carcinogenic activity of 4-dimethylaminoazobenzene is markedly inhibited by high levels of dietary riboflavin. Similarly, rats fed high levels of riboflavin have lower hepatic levels of bound dye than rats fed amounts of riboflavin allowing rapid tumor formation. The rate of tumor induction is also dependent on the level of dye fed, and the

[1] R. R. Brown, J. A. Miller, and E. C. Miller, unpublished data.

level of hepatic bound dye increases with increasing dietary levels of dye.

In studies on the structural requirements for carcinogenic activity, several series of azo dyes with widely different activities have been found (17, 18, 22, 35, 36). Thus, at least one N-methyl group is required for high carcinogenic activity, and, similarly, at least one N-methyl group is required for the formation of appreciable levels of protein-bound dye. N-mono- or di-alkyl compounds *not* containing a methyl group have not yielded tumors, and only low levels of bound dye can be found in the livers of rats fed these compounds. Similarly, 4-aminoazobenzene is noncarcinogenic in our tests and yields very low levels of bound dye *in vivo*. However, as reported in an earlier session, significant carcinogenic activity has recently been found for 3′-methyl- and 4′-fluoro-4-aminoazobenzene (19). These carcinogenic activities have been correlated with the extent to which the compounds are N-methylated *in vivo* and with the amount of bound dye formed from these N-methyl metabolites. In another series the compounds differ only in the position of a

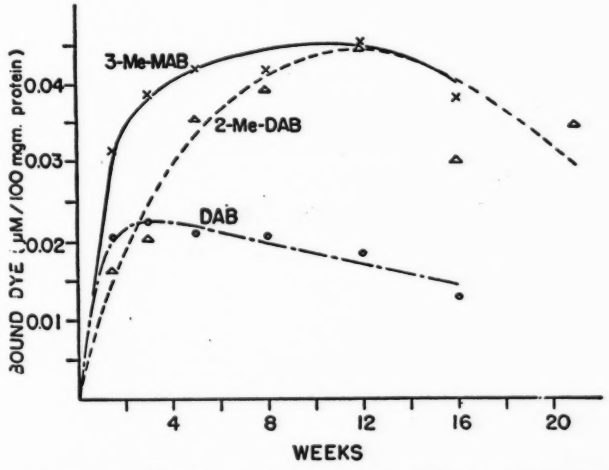


CHART 4.—The levels of protein-bound dye in the livers of rats fed 4-dimethylaminoazobenzene, 2-methyl-4-dimethylaminoazobenzene, or 3-methyl-4-monomethylaminoazobenzene (15). (Reprinted from *Cancer Research*, 9:336–43, 1949.)

single ring methyl substituent; yet the activities vary greatly. The micromolar levels of bound dye found after the feeding of each of these dyes for various periods of time are plotted in Charts 3 and 4 (15). When 4-dimethylaminoazobenzene or its 2′-, 3′-, or 4′-methyl derivatives were fed, the initial rates of increase and the maximum levels of bound dye correlated directly with the carcinogenic activities of the dye fed; but the times at which the maximum levels of bound dye were reached were inversely related to the carcinogenic activities. When the essentially inactive dyes 2-methyl-4-dimethylaminoazobenzene or 3-methyl-4-monomethylaminoazobenzene were fed, the shape of the bound dye curves was that characteristic of a very weak carcinogen, but the levels of bound dye were higher than with other weak carcinogens. However, recent preliminary studies indicate that the *in vivo* turnover rates of the protein-bound dyes derived from the 2- and 3-methyl dyes are lower than those for the bound dyes derived from 4-dimethylaminoazobenzene or its

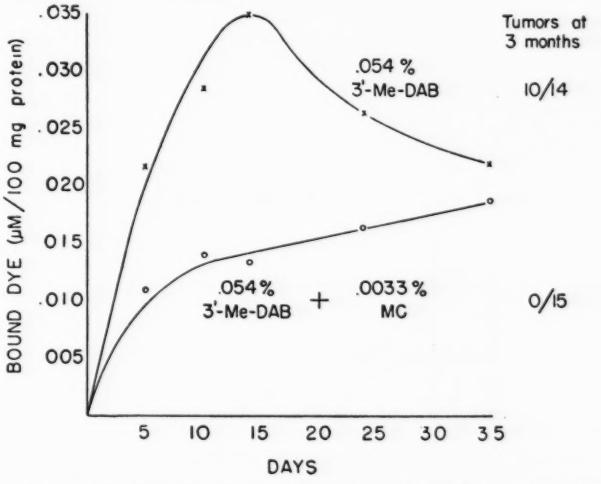


CHART 5.—The levels of protein-bound dye in the livers of rats fed diets containing 0.054 per cent of 3′-methyl-4-dimethylaminoazobenzene with or without 0.0033 per cent of 20-methylcholanthrene. The tumor incidences for rats fed these diets for 3 months are expressed as the number of rats with liver tumors over the number of rats alive at this time. (Reprinted from *Advances in Cancer Research*, Vol. 1, with the permission of the publishers.)

other ring-methyl derivatives, and these lower turnover rates appear to be responsible for the higher levels of bound dye found at any one time.

Last year at these meetings, Richardson and Cunningham (28) reported that the addition of a low level of 20-methylcholanthrene to a diet containing 3′-methyl-4-dimethylaminoazobenzene strongly inhibited the induction of liver tumors by the azo dye. This treatment also depresses the rate of bound dye accumulation and alters the shape of the curve so that it resembles that yielded by a weakly active dye (Chart 5) (14). Finally, in an earlier session of these meetings Salzberg and Griffin (30) reported that the livers of rats made diabetic with alloxan were resistant to the carcinogenic action of 3′-methyl-4-dimethylaminoazobenzene and contained much less bound dye than the livers of control rats susceptible to tumor induction.

To us one of the most intriguing observations is that, while the livers of rats fed the azo dyes continuously always contain some bound dye, the tu-

mors arising in these livers do not contain detectable quantities. The tumors do, however, contain appreciable levels of the free dyes, so lack of penetration of the dyes into the tumors does not seem to be responsible for the lack of bound dye (see also discussion in [20]). This finding suggested to us that the carcinogenic dyes, through combination with certain proteins, might cause their gradual deletion from the liver cells. And, finally, cells might arise which had only very low levels or none

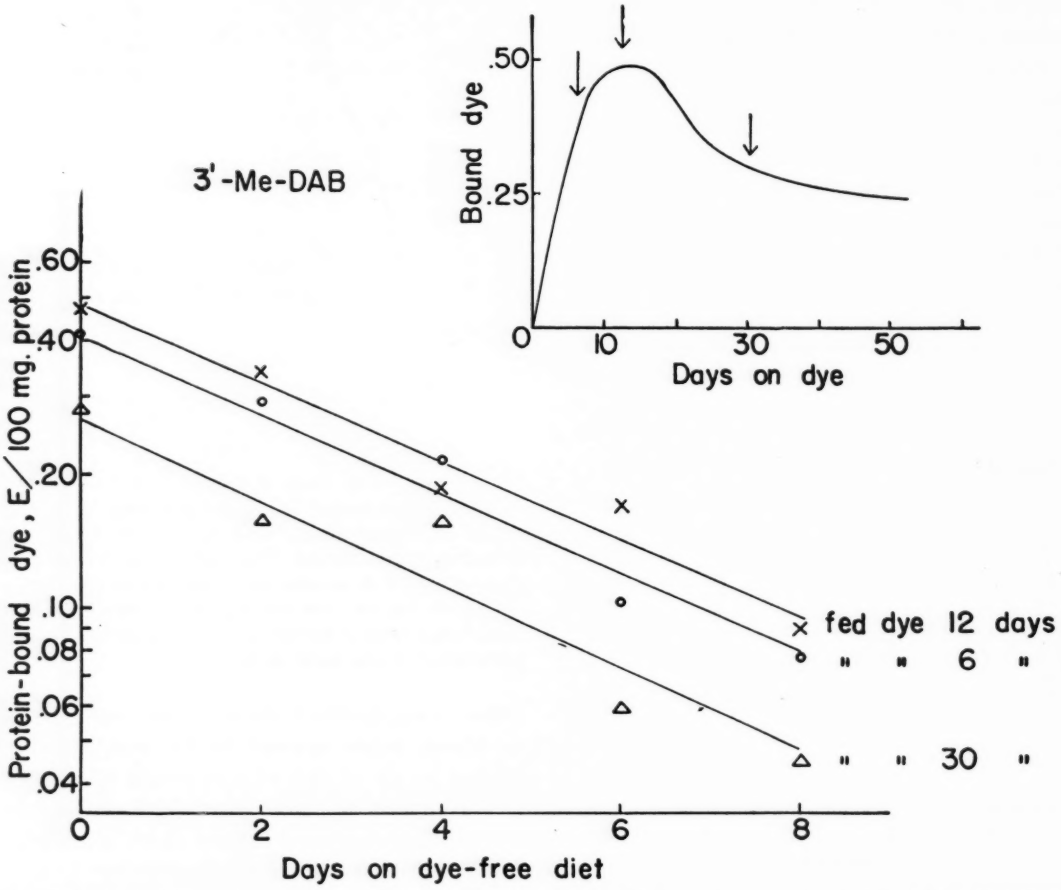


CHART 6.—The rates of disappearance of protein-bound dye from the livers of rats transferred to a dye-free diet after being fed 0.054 per cent of 3'-methyl-4-dimethylaminoazobenzene for 6, 12, or 30 days.

of these particular proteins. The shape of the bound dye curves obtained with the carcinogenic dyes is consonant with this view, particularly since the shape apparently *cannot* be explained by an increased rate of destruction of the bound dye as the time of dye-feeding increases (Chart 6). Thus, when the rate of disappearance of bound dye on transferring rats from a dye-containing to a basal diet was determined, the rate of loss was approximately the same whether the disappearance curve was started while the bound dye level in the liver was increasing, at a plateau, or decreasing. The half-life for the bound dye was approximately $3\frac{1}{2}$ days or about the same as for the average liver protein of normal rats (37). In view of this similarity in half-lives and our inability to demonstrate metabolism of the protein-bound dyes *in vitro* under conditions suitable for the metabolism of the free dyes, the dyes appear to be relatively resistant to metabolism while they are bound to the liver proteins.

Since fractionation studies (27) showed that approximately one-half of the bound dye in the livers was associated with the soluble proteins, analyses of the distribution of the bound dye among the electrophoretic components of the soluble protein fraction were made by Sorof *et al.* (34). When fractions were removed in sequence from both the ascending and descending limbs, the amount of bound dye in each fraction was found to be approximately related to the amount of the slowly moving *h* component in that fraction (Chart 7). Approximately 80 per cent of the bound dye appeared to be associated with a fraction which comprised only 8–15 per cent of the soluble proteins. Furthermore, on analysis of the electrophoretic

components of the soluble proteins from tumors induced by the azo dyes, Sorof and Cohen (33) also found only about 10 per cent as much of this *h* fraction in the tumors as in normal liver. These findings lend strong support to the idea that the binding of dye by certain liver proteins might so alter the proteins that they are eventually lost to the cell.

At present we feel that all these findings offer support for a protein or enzyme-deletion theory of azo dye carcinogenesis. Thus, the dye metabolite may combine with certain liver proteins essential for the control of growth but not for the life of the cell. If one further assumes that such binding could in some way prevent or hinder the synthesis of these proteins or induce the synthesis of somewhat altered proteins through faulty duplication, then eventually cell generations might arise which were completely lacking in one or more normal growth control proteins. Such cells could only respond to continued nutrition by continued growth and would thus be tumor cells.

Such a theory of the mechanism of cancer induction may also be applicable to other types of

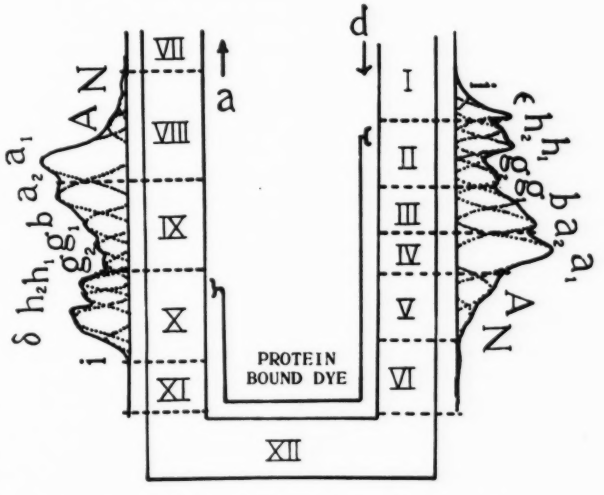


CHART 7.—The distribution of protein-bound dye among the electrophoretic fractions of the soluble liver proteins from dye-fed rats (34). (Reprinted from *Cancer Research*, **11**:383–87, 1951.)

chemical carcinogens. Thus, we found that the skin of mice painted with the carcinogenic hydrocarbon 3,4-benzpyrene contains derivatives of the hydrocarbon chemically bound to the epidermal protein (12). These derivatives, detected by their strong blue fluorescence, were found only in the epidermis and not in a number of other tissues which were examined. The epidermis is the major site of tumor formation under these conditions. Furthermore, when mice painted with benzpyrene were kept in the dark, the epidermal protein contained considerably more protein-bound fluorescence than when the mice were kept in bright light (Chart 8). This observation correlates with the finding that mice painted with benzpyrene and kept in the dark have a higher incidence of skin tumors than those kept in bright light (3, 23). It also appears to rule out the possibility that photooxidation products of the hydrocarbon are involved in the reaction. Following a single application of benzpyrene, the maximum level of protein-bound fluorescence was reached after approxi-

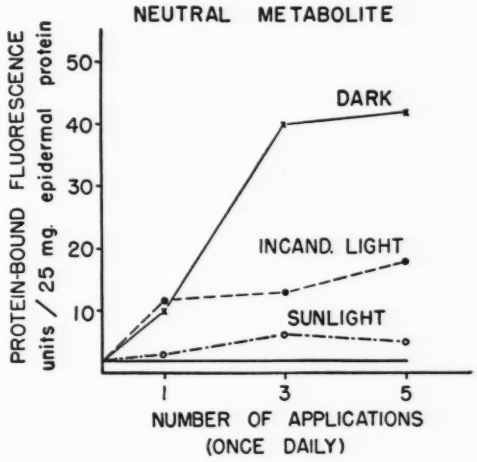


CHART 8.—The levels of the protein-bound neutral derivative derived from 3,4-benzpyrene in the epidermis of mice treated daily with the hydrocarbon and maintained in darkness, in direct sunlight, or under incandescent lamps. The solid line at the bottom of the graph indicates the protein-bound fluorescence obtained from mice treated only with benzene (12). (Reprinted from *Cancer Research*, **11**:100–108, 1951.)

mately 1 day (Chart 9), and the amount then gradually decreased until it was just detectable at 2 weeks. Using C^{14}-labeled 1,2,5,6-dibenzanthracene, Wiest and Heidelberger (39) obtained a similar curve for the amount of protein-bound C^{14} in the epidermis following a single application to the skin (Chart 10). Protein-bound C^{14} was also found at the sites of injection of the labeled dibenzanthracene into the submaxillary glands or subcutaneous tissue of mice (9, 39). As in the case of the azo dyes these hydrocarbon derivatives do not appear to be found through the nucleic acids in these protein preparations (12, 39). Finally, both Weisburger *et al.* (38) and the present authors[2] have found protein-bound C^{14} in the livers of rats given 2-acetylaminofluorene-9-C^{14}. The liver is one of the major sites of tumor formation under these conditions. As in the case of the protein-bound aminoazo dyes the major share of the protein-bound C^{14} following the administration of

[2] We are greatly indebted to Dr. Charles Heidelberger for the 2-acetylaminofluorene-9-C^{14}.

the labeled 2-acetylaminofluorene is found in the soluble protein fraction (Table 2). While the studies on the protein-bound derivatives of the polycyclic hydrocarbons and 2-acetylaminofluorene are much less complete than in the case of the azo dyes, they do point to the possible importance

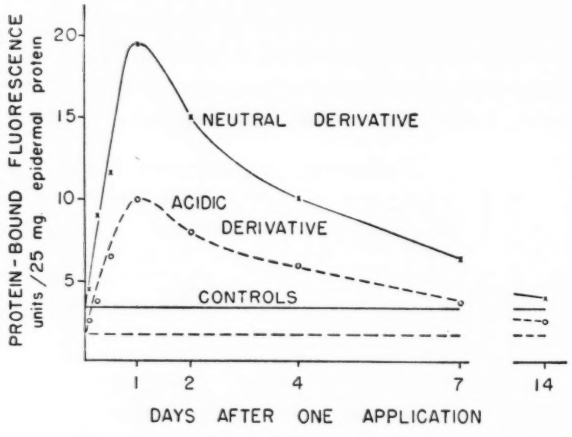


CHART 9.—The levels of fluorescent protein-bound derivatives of 3,4-benzpyrene in the epidermis of mice given a single topical application of the carcinogen at zero time. The solid and broken lines at the bottom of the graph indicate the fluorescence in the neutral and acidic fractions from benzene-treated epidermis (12). (Reprinted from *Cancer Research*, **11**:100–108, 1951.)

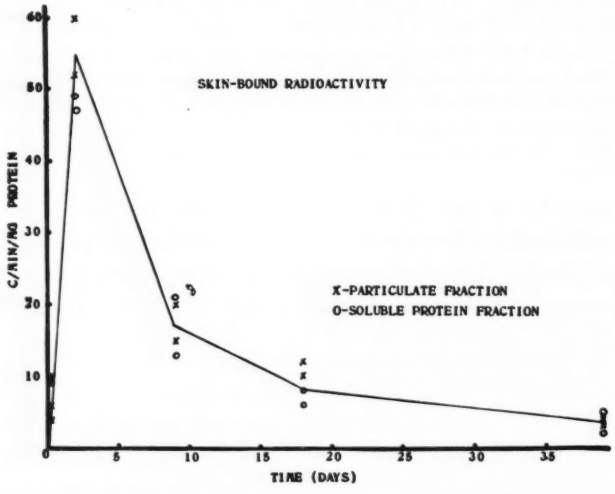


CHART 10.—The levels of protein-bound C^{14} in the particulate and soluble protein fractions from the epidermis of mice given a single topical application of 1,2,5,6-dibenzanthracene-9,-10-C^{14} at zero time (from Wiest and Heidelberger [39]).

of protein-carcinogen complexes in tumorigenesis by these agents.

And in a speculative manner we might consider the sarcogenic activity of Light Green F. S. (8, 31). Recently, Braun (2), using crystal violet as an example, found that triphenylmethane dyes react with protein –SH groups to yield compounds of the type depicted in Chart 11. By analogy it might be expected that Light Green F. S. would react similarly. Such a reaction, if it took place *in vivo*, might be of considerable importance in the induction of sarcomas by the dye.

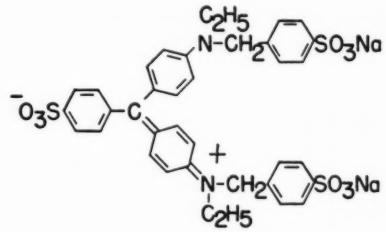
This general theory of carcinogenesis through protein- or enzyme-deletion or alteration could

TABLE 2

THE DISTRIBUTION OF PROTEIN-BOUND C^{14} AMONG THE LIVER FRACTIONS FROM A RAT FED 2-ACETYLAMINOFLUORENE-9-C^{14} FOR 27 DAYS

Fraction	Specific activity (counts/min/mg protein)	Total activity in the liver (counts/min)
Whole liver	113	77,000
Nuclei	91	18,000
Large granules	80	13,000
Small granules	96	13,000
Supernatant fluid	108	28,000

During this period, the 200-gm. rat, a male, consumed 108 mg. of 2-acetylaminofluorene-9-C^{14} with an activity of 1×10^8 counts/min/mg of compound. The differential centrifugations were carried out as described in (27).



Light Green F.S.; F.D.&C. Green No. 2
(sarcogen for rats: Schiller '37; Harris '47)

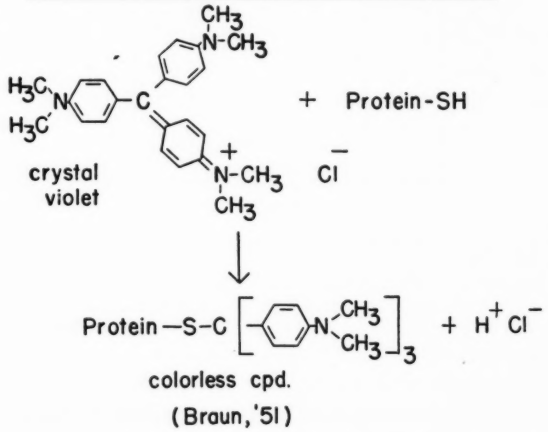


CHART 11.—The formula of the sarcogen Light Green F. S. and a reaction by which triphenylmethane dyes react with proteins nonenzymatically *in vitro*.

also be extended to include other types of carcinogens. Thus, physical carcinogens such as rays from radioactive elements or ultraviolet light could act by damaging, in some cells, key proteins required for the control of growth but not essential for

growth itself. Again, carcinogenic viruses might be considered to compete so effectively for cell nutrients that cells deficient in certain of the normal growth-control proteins might be formed. From this standpoint a carcinogenic virus need not necessarily be a "continuing cause," but instead it could be only an "initiating cause" and thereafter merely reside within the tumor cells.

In any of these processes it is evident that large numbers of cells in the target tissue are damaged by the carcinogenic agent. However, of the damaged cells, some may lack enzymes vital to life itself and thus die. Others may be deficient in some respects but may still be able to survive and not proliferate in an uncontrolled fashion. And still others may be altered in just the right ways to give cells capable of continued growth but relatively free of certain growth-controlling factors. In an appropriate environment, such cells might then proliferate to yield tumor masses.

REFERENCES

1. BOYLAND, E. Different Types of Carcinogens and Their Possible Modes of Action: *A Review*. Cancer Research, **12**: 77–84, 1952.
2. BRAUN, A. D. Interaction of Proteins with Triphenylmethane Dyes. Biokhimiya, **16**:399–409, 1951. "Abstr." Chem. Abstr., **46**:2103, 1952.
3. DONIACH, I., and MOTTRAM, J. C. On the Effect of Light upon the Incidence of Tumours in Painted Mice. Am. J. Cancer, **39**:234–40, 1940.
4. DONIACH, I.; MOTTRAM, J. C.; and WEIGERT, F. The Fluorescence of 3,4-Benzpyrene *in vivo*. Part I. The Distribution of Fluorescence at Various Sites, Especially the Skin of Mice. Brit. J. Exper. Path., **24**:1–9, 1943.
5. FIESER, L. F. Carcinogenic Activity, Structure, and Chemical Reactivity of Polynuclear Aromatic Hydrocarbons. Am. J. Cancer, **34**:37–124, 1938.
6. ———. Production of Cancer by Polynuclear Hydrocarbons. In L. F. FIESER *et al.*, Cause and Growth of Cancer, pp. 1–27. Philadelphia: University of Pennsylvania Press, 1941.
7. FRAENKEL-CONRAT, H., and OLCOTT, H. S. Reaction of Formaldehyde with Proteins. VI. Cross-Linking of Amino Groups with Phenol, Imidazole, or Indole Groups. J. Biol. Chem., **174**:827–43, 1948.
8. HARRIS, P. N. Production of Sarcoma in Rats with Light Green SF. Cancer Research, **7**:35–36, 1947.
9. HEIDELBERGER, C., and WEISS, S. M. The Distribution of Radioactivity in Mice Following Administration of 3,4-Benzpyrene-5-C^{14} and 1,2,5,6-Dibenzanthracene-9,10-C^{14}. Cancer Research, **11**:885–91, 1951.
10. HENDRY, J. A.; HOMER, R. F.; ROSE, F. L.; and WALPOLE, A. L. Cytotoxic Agents: III, Derivatives of Ethyleneimine. Brit. J. Pharmacol. and Chemotherapy, **6**:357–410, 1951.
11. MACDONALD, J. C.; PLESCIA, A. M.; MILLER, E. C.; and MILLER, J. A. Studies on the Metabolism of Various N-Methyl-C^{14}-substituted Aminoazo Dyes. Cancer Research, **12**:280, 1952.
12. MILLER, E. C. Studies on the Formation of Protein-bound Derivatives of 3,4-Benzpyrene in the Epidermal Fraction of Mouse Skin. Cancer Research, **11**:100–108, 1951.
13. MILLER, E. C., and MILLER, J. A. The Presence and Significance of Bound Aminoazo Dyes in the Livers of Rats Fed *p*-Dimethylaminoazobenzene. Cancer Research, **7**: 468–80, 1947.
14. MILLER, E. C.; MILLER, J. A.; and BROWN, R. R. On the Inhibitory Action of Certain Polycyclic Hydrocarbons on Azo Dye Carcinogenesis. Cancer Research, **12**:282–83, 1952.
15. MILLER, E. C.; MILLER, J. A.; SAPP, R. W.; and WEBER, G. M. Studies on the Protein-bound Aminoazo Dyes Formed *in vivo* from 4-Dimethylaminoazobenzene and Its C-Monomethyl Derivatives. Cancer Research, **9**:336–43, 1949.
16. MILLER, E. C.; PLESCIA, A. M.; MILLER, J. A.; and HEIDELBERGER, C. The Metabolism of Methylated Aminoazo Dyes. I. The Demethylation of 3′-Methyl-4-dimethyl-C^{14}-aminoazobenzene *in Vivo*. J. Biol. Chem., **196**:863–74, 1952.
17. MILLER, J. A., and BAUMANN, C. A. The Carcinogenicity of Certain Azo Dyes Related to *p*-Dimethylaminoazobenzene. Cancer Research, **5**:227–34, 1945.
18. MILLER, J. A., and MILLER, E. C. The Carcinogenicity of Certain Derivatives of *p*-Dimethylaminoazobenzene in the Rat. J. Exper. Med., **87**:139–56, 1948.
19. ———. On the Carcinogenicity and Methylation *in vivo* of 4-Aminoazobenzene and Its 3′-Methyl and 4′-Fluoro Derivatives in the Rat. Cancer Research, **12**:283, 1952.
20. ———. The Carcinogenic Aminoazo Dyes. Advances in Cancer Research, Vol. I. New York: Academic Press, 1952 (in press).
21. MILLER, J. A.; SAPP, R. W.; and MILLER, E. C. The Absorption Spectra of Certain Carcinogenic Aminoazo Dyes and the Protein-bound Derivatives Formed from These Dyes *in Vivo*. J. Am. Chem. Soc., **70**:3458–63, 1948.
22. ———. Carcinogenic Activities of Certain Halogen Derivatives of 4-Dimethylaminoazobenzene in the Rat. Cancer Research, **9**:652–60, 1949.
23. MORTON, J. J.; MIDER, G. B.; LUCE-CLAUSEN, E. M.; and MAHONEY, E. B. The Effect of Visible Light on the Development in Mice of Skin Tumors and Leukemia Induced by Carcinogens. Cancer Research, **11**:559–61, 1951.
24. MUELLER, G. C., and MILLER, J. A. The Oxidative Demethylation of N-Methylaminoazo Dyes by Rat Liver Homogenates. Cancer Research, **11**:271, 1951.
25. POTTER, V. R. Biological Energy Transformations and the Cancer Problem. Advances in Enzymology, **4**:201–56. New York: Interscience Publishers, Inc., 1944.
26. POTTER, V. R.; PRICE, J. M.; MILLER, E. C.; and MILLER, J. A. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. III. Effects on Succinoxidase and Oxalacetic Acid Oxidase. Cancer Research, **10**:28–35, 1950.
27. PRICE, J. M.; MILLER, E. C.; MILLER, J. A.; and WEBER, G. M. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. II. 3′-Methyl-, 2′-Methyl-, and 2-Methyl-4-dimethylaminoazobenzene, 3-Methyl-4-monomethylaminoazobenzene, and 4′-Fluoro-4-dimethylaminoazobenzene. Cancer Research, **10**:18–27, 1950.
28. RICHARDSON, H. L., and CUNNINGHAM, L. The Inhibitory Action of Methylcholanthrene on Rats Fed the Azo Dye 3′-Methyl-4-dimethylaminoazobenzene. Cancer Research, **11**:274, 1951.
29. ROSKELLY, R. C.; MAYER, N.; HORWITT, B. N.; and SALTER, W. T. Studies in Cancer. VII. Enzyme Deficiency in Human and Experimental Cancer. J. Clin. Investigation, **22**:743–51, 1943.
30. SALZBERG, D. A., and GRIFFIN, A. C. Inhibition of Azo

Dye Carcinogenesis in the Alloxan-diabetic Rat. Cancer Research, **12**:294, 1952.

31. Schiller, W. Rat Sarcoma Produced by the Injection of the Dye, Light Green F. S. Am. J. Cancer, **31**:486–90, 1937.
32. Schneider, W. C. Phosphorus Compounds in Animal Tissues. I. Extraction and Estimation of Desoxypentose Nucleic Acid. J. Biol. Chem., **161**:293–303, 1945.
33. Sorof, S., and Cohen, P. P. Electrophoretic and Ultracentrifugal Studies on the Soluble Proteins of Various Tumors and of Livers from Rats Fed 4-Dimethylaminoazobenzene. Cancer Research, **11**:376–82, 1951.
34. Sorof, S.; Cohen, P. P.; Miller, E. C.; and Miller, J. A. Electrophoretic Studies on the Soluble Proteins from Livers of Rats Fed Aminoazo Dyes. Cancer Research, **11**: 383–87, 1951.
35. Sugiura, K. The Carcinogenicity of Certain Compounds Related to *p*-Dimethylaminoazobenzene. Cancer Research, **8**:141–44, 1948.
36. Sugiura, K.; Halter, C. R.; Kensler, C. J.; and Rhoads, C. P. Observations on Rats Fed with Compounds Related to Dimethylaminoazobenzene. Cancer Research, **5**:235–38, 1945.
37. Tarver, H. The Metabolism of Amino Acids and Proteins, pp. 769–890. In D. M. Greenberg, Amino Acids and Proteins. Springfield: Charles C. Thomas Co., 1951.
38. Weisburger, J. H.; Weisburger, E. K.; Morris, H. P.; and Sober, H. A. Studies on the Metabolism of 2-Acetylaminofluorene-9-C^{14}. Cancer Research, **12**:305, 1952.
39. Wiest, W. G., and Heidelberger, C. Studies on the Interaction of Labeled Carcinogenic Hydrocarbons with Tissue Components. Cancer Research, **12**:308, 1952.

Paper No. 4 in the Symposium on Immunogenetics and Carcinogenesis

Chemical and Immunological Properties of Carcinogen-Protein Conjugates

HUGH J. CREECH

(The Institute for Cancer Research and the Lankenau Hospital Research Institute, Philadelphia 11, Pa.)

It is obvious from the remarks of the preceding speakers that steady progress toward a more complete understanding of the many complex chemical and biological factors involved in tumor transplantation and in carcinogenesis is being made by the application of a variety of experimental procedures. Also, it seems likely that the interesting observations on the *in vivo* combination of carcinogens with proteins just presented by Dr. E. Miller eventually will be found to be clearly related to those on the synthetic carcinogen-protein conjugates which I am privileged to review today. This work requires the knowledge and technics of several scientific disciplines, and I have been fortunate in having many capable collaborators since this research was started in 1935. Without their enthusiasm and co-operation, the problem probably would have been abandoned long ago. In addition to the financial and scientific support received at the University of Toronto, Harvard University, the University of Maryland, and the Institute for Cancer Research in Philadelphia, I also wish to acknowledge with appreciation grants-in-aid given by the International Cancer Research Foundation and the American Cancer Society acting through the Committee on Growth of the National Research Council.

The goal of the research is to inhibit carcinogenesis by immunological methods. There seems to be no theoretical reason why this cannot be done. From the practical standpoint, however, it was evident from the beginning that even partial attainment of the final aims would require patience and continuous over-all optimism. It has been a source of satisfaction to us that developments of practical importance to other problems and to other workers have emerged from time to time during the course of the experimentation.

The attack on the problem was based upon two separate observations. The first of these was the brilliant work of Landsteiner and his group (30), who discovered that the chemical attachment of a relatively complex, foreign, organic compound to a protein produced a conjugate which showed a new type of antigenic activity called prosthetic group, determinant group, or haptenic group activity. These workers demonstrated that antisera, elicited by the injection of such conjugates into animals, reacted serologically both with the protein component and with the foreign prosthetic group of the test antigens. Moreover, the reactivity of such antisera was not confined solely to protein conjugates. It was shown that these antisera would react *in vitro* and *in vivo* with the foreign compound alone or with a soluble, nonantigenic conjugate made by attaching the prosthetic group to an amino acid or peptide. The second set of observations consisted of the demonstration by Kennaway, Cook, Hieger, *et al.* (3) in England and Fieser, Shear, and others (19, 21) in this country of the carcinogenicity of a variety of carefully characterized chemicals of the polynuclear aromatic hydrocarbon type. Concurrently, there emerged the possibility that structurally related compounds, elaborated in animals as a result of abnormal metabolism, might be responsible for the production of certain spontaneous tumors.

It seemed of interest, therefore, to determine whether the combination of various chemical carcinogens with proteins would produce antigens which, upon injection into animals, would elicit antisera capable of preventing carcinogenesis. From the practical point of view, the obvious procedure was to combine several polynuclear aromatic hydrocarbons with proteins, study their antigenic properties, and determine their potentialities in protecting animals against the carcinogenic activity of known chemicals. Preliminary investigations (9, 10, 22) conducted with Drs. W. R. Franks and F. G. Banting at the University of Toronto in the pe-

riod 1935–38 suggested that protection against carcinogenesis was possible by such procedures. It seemed worth while, therefore, to continue the investigation with the use of carcinogen-protein conjugates which could be more carefully characterized chemically and antigenically than the earlier preparations. Several biological difficulties still have to be overcome before a conclusive demonstration of immunological protection will be possible. In the event that such can be done, and if some investigator should isolate an endogenous carcinogenic chemical from tumors or other tissues, or if carcinogenesis due to an exogenous chemical should assume great significance, the application of immunological procedures conceivably might become of practical importance. During the course of this work, it was encouraging to note that other investigators were able to immunize animals against physiologically active chemicals such as histamine (32), thyroxine (2), and aspirin (1) by similar methods.

The first problem was the development of suitable procedures for combining carcinogens with proteins. Because of the chemical nature of the amino derivatives of the carcinogens available in the early stages of our research, it was not possible to employ the widely used azo linkage. Of the several other possibilities, recently analyzed by Herriott (27), a choice was made of the carbamido linkage -NHCONH- employed by Hopkins and Wormall (28). Subsequent work has demonstrated that this linkage has many advantages. Isocyanates of the carcinogens were found to combine with proteins under mild experimental conditions. The reaction involves the E-amino groups of the lysine side chains of the protein as follows. $ArNCO + H_2N\text{-protein} \rightarrow ArNHCONH\text{-protein}$. Zein, which is deficient in lysine, failed to react with the isocyanates under extremely favorable conditions (13). While in Professor Fieser's laboratory at Harvard University, I synthesized isocyanates of 1,2-benzanthracene, 1,2,5,6-dibenzanthracene (20), 10-methyl-1,2-benzanthracene, and 3,4-benzpyrene (7) and combined them with proteins and amino acids (11, 12, 13, 20). Later, an isocyanate of 9,10-dimethyl-1,2-benzanthracene was prepared by Dr. Mayo Smith (33), and recently Dr. R. M. Peck has synthesized isocyanates of 2-acetylaminofluorene, 4-dimethylaminostilbene, and its 2′-methyl analog (31). All these isocyanates have been found to react readily with proteins in an aqueous dioxane medium buffered at pH 8–10 and cooled to 0 to $-10°$. Other reaction media were inferior (20). An accurate spectrophotometric method to determine the amount of combined carcinogen was developed by Dr. R. N. Jones (29) and will be described later in the paper.

With the advice and help of Professor E. J. Cohn and Dr. T. L. McMeekin of the Harvard Medical School, I learned the necessary technics for the manipulation of the starting proteins and the resultant conjugates. It was soon discovered that the most satisfactory proteins were the serum albumins, because they readily withstand the experimental conditions and because they have a high content of lysine (11, 12). Attempts to introduce an appreciable amount of carcinogen into egg albumin or into the serum globulins usually resulted in serious denaturation which made accurate spectrophotometric analysis impossible and rendered difficult and unreliable any interpretations of their chemistry and serology (13). Purification of the conjugate was accomplished by dialysis, centrifugation, and fractionation with ammonium sulfate by the rotating cellophane membrane technic; these procedures removed the majority of adsorbed material. A final precipitation with acetone was usually necessary to remove the last traces of impurities. The conjugates then were found to retain a constant prosthetic group content following repeated and different chemical treatments. The purified serum albumin conjugates dissolved readily in distilled water, giving optically clear solutions usually at a protein concentration of 1–2 per cent. The amount of carcinogen prosthetic group introduced into the protein could be varied at will up to a maximum by changing the experimental conditions of the coupling process. The use of high percentages of dioxane and of isocyanate, along with an alkaline pH in the reaction medium, increased the extent of combination of the carcinogen with the protein. However, if the dioxane level was increased above 45 per cent or if the pH was raised above 10, there was considerable denaturation of the protein even at $-10°$. Based on the lysine content, it should be possible to introduce 66 prosthetic groups into a molecule of serum albumin. The maximum amount of polycyclic aromatic hydrocarbon introduced without significant denaturation of the albumin was 38 1,2-benzanthryl groups. The isocyanate of 1,2-benzanthracene is much more soluble in dioxane than are those of 3,4-benzpyrene or 1,2,5,6-dibenzanthracene, in which cases the maximum numbers of groups combined with serum albumin have been 19 and 21, respectively. The 4′-isocyanate of 2′-methyl-4-dimethylaminostilbene is even more reactive than the polycyclic isocyanates, and Dr. Peck has recently prepared serum albumin

conjugates containing 55 and 62 groups of this (type 17).

The polycyclic aromatic hydrocarbons display characteristic absorption spectra with several sharp maxima in the region of 260–380 mμ. In order to determine the influence of the carbamido linkage and the solvent, studies were made of the absorption spectra of water-soluble reference compounds prepared by combining the hydrocarbon isocyanates with amino acids such as E-aminocaproic acid (11, 12). The intensities of the maxima were identical to those of the hydrocarbon at wave-lengths greater than 325 mμ. In the case of the protein conjugates, accurate analysis of the amount of hydrocarbon prosthetic group was possible only at wave-lengths greater than 325 mμ because of the interfering absorption of the protein, which has a maximum at 277 mμ owing to the tyrosine, phenylalanine, and tryptophan content (11). The prosthetic chromophore absorbs independently, because it is separated from the protein molecule by four methylene groups. The number of prosthetic groups per molecule of protein was calculated by determining the amount of nitrogen in the conjugate by micro-Kjeldahl analysis and by comparing the observed intensity of absorption of its hydrocarbon component with that of the reference compound.

Although the spectrum of the fluorene component of the conjugates has its broad maximum at 294 mμ, the intensity of absorption is sufficiently high that spectrophotometric analysis is possible in spite of protein absorption at that wavelength (17). Considerable difficulties in interpretation were encountered with the dimethylaminostilbenes because of *trans-cis* isomerism, which results in a shift of the maxima to shorter wavelengths preceded by, and accompanied by, an appreciable decrease in the intensity of absorption. When aqueous solutions of the amino acid reference compounds were exposed to light, there was a shift in the wave-length of the maximum from 358 mμ to 330 mμ, accompanied by a 50 per cent drop in intensity within a few minutes. Aqueous solutions of the stilbene-protein conjugates also have been found to undergo *trans-cis* shifts, although the process in this case required a matter of days of constant exposure to light before appreciable changes occurred. Accurate analysis of the stilbene content of the amino acid and protein conjugates was accomplished by dissolving them in 1:1 dioxane-water and by protecting the solutions from light until the spectrophotometric analysis was made (17). Under such conditions, the amino acid conjugates and the protein conjugates were found to exist in the *trans* configuration with the maximum located at 355–358 mμ.

The degree of homogeneity of the conjugates has always been a question of some concern. Two different procedures have been utilized to obtain information on this point. The first method involved attempted fractionation of conjugates containing the 1,2-benzanthryl prosthetic group (12). Spectrophotometric analysis of various fractions removed at different levels of ammonium sulfate concentration showed in all cases a similar amount of prosthetic group per gram of protein. On the other hand, serum albumin could be separated sharply from the conjugates by ammonium sulfate fractionation. This indicates rather uniform coupling of the isocyanate with the protein with the formation of conjugates in which the majority of protein molecules contain close to the average number of prosthetic groups. Recently, Dr. Gail Miller has conducted electrophoretic studies on several bovine serum albumin conjugates containing different amounts of 4-dimethylaminostilbene and 2′-methyl-4-dimethylaminostilbene. Highly significant differences were observed in the mobilities of the preparations. Bovine serum albumin had a mobility of 6.7×10^{-5} cm²/volt-sec, whereas, under identical experimental conditions, conjugates containing 20, 37, and 62 stilbenyl groups had mobilities of 7.8, 9.7, and 10.4×10^{-5} cm²/volt-sec. Since the electrophoretic diagrams revealed no abnormal spreading of the boundaries, the conjugates appear to be homogeneous. Moreover, when a mixture of nine parts of the conjugate containing 37 groups and one part of the conjugate containing 62 groups was subjected to electrophoresis, there was a definite resolution of the components of the mixture. Sedimentation studies were inconclusive because of the high degree of aggregation shown by the conjugates.

A property of the conjugates which has been of considerable interest to other workers is the intense fluorescence which they display in ultraviolet light. Dependent on the prosthetic group, aqueous solutions containing only a few micrograms of the conjugates exhibit green, blue-green, blue, or purple-blue fluorescence. This property has been used routinely in our serological studies and occasionally to trace the conjugate after injection into animals. In a collaborative project with Dr. A. H. Coons of the Harvard Medical School (4, 5), we found that these isocyanates also could be coupled with various purified antibodies to give fluorescent conjugates. When the conjugates were prepared under very mild experimental conditions, they were found to retain their original serological

specificity and potency which rendered them useful for the demonstration of pneumonococcal antigen in tissue. Isocyanates of fluorescein were found particularly advantageous for this work because of the green fluorescence of the resultant conjugates. Recently, Dr. Coons has extended this research to the location of antigens of rickettsiae and mumps virus in tissue cells (6).

As a result of the preceding chemical work, we were able to begin our immunological studies with rather highly qualified antigens. We had started with pure single proteins rather than with a mixture of proteins, we had combined them through the E-amino groups with well characterized carcinogens in such a way that denaturation was at a minimum, we were able to determine the prosthetic group content accurately, we had preparations which differed considerably from one another in the number of prosthetic groups per molecule, and we had evidence pointing toward the homogeneity of the preparations. Furthermore, the carbamido linkage has been found to be very stable. Hydrocarbon conjugates stored under ammonium sulfate for 9 years were readily soluble in distilled water after dialysis and, when analyzed spectrophotometrically both before and after acetone precipitation, showed a decrease in prosthetic group content of only 8 per cent. One aqueous solution which had been stored in the refrigerator at a concentration of 30 mg of protein/ml was bacteriologically sterile, contained no precipitate, and showed a decrease in prosthetic group content of less than 20 per cent in a 7-year period. In addition, the presence of the fluorescent prosthetic group was of considerable aid in the serological work.

TABLE 1

HYDROCARBON SERIES

MG. OF AG.-AB. PPT. PER ML. ANTISERUM

LINE	Antiserum	Antigen	Mg. antigen added 0.2	0.5	1.0
1	HSA	HSA	0.95	*1.20*	0.63
2	HSA	bz-HSA-38		1.09	*1.35*
3	bz-HSA-16	HSA	*0.60*		
4	bz-HSA-16	bz-HSA-16	1.50	*2.00*	1.18
5	bz-HSA-38	HSA	*0.34*	0.28	
6	bz-HSA-38	bz-HSA-38	0.49	*0.51*	
7	bz-HSA-16	bz-BovA-15	*0.35*	0.32	
8	bz-HSA-16	bz-USA-29	0.32	*0.58*	0.45
9	bz-HSA-38	bz-BovA-15	0.48	*0.66*	0.34
10	bz-HSA-38	BP-BovA-19			*0.43*
11	bz-USA-29	bz-HSA-16		0.23	*0.26*
12	bz-USA-29	bz-HSA-38		*0.24*	
13	bz-USA-29	bz-BovA-15	0.30	*0.34*	0.19
14	bz-USA-29	Dba-BovA-20			*0.12*
15	bz-USA-29	BP-BovA-19			*0.19*

Proteins: HSA = horse serum albumin; BovA = bovine serum albumin; USA = human serum albumin.

Prosthetic groups: bz = 1,2-benzanthryl-10-carbamido; BP = 3,4-benzpyrenyl-5-carbamido; Dba = 1,2,5,6-dibenzanthryl-9-carbamido. bz-HSA-16 represents a horse serum albumin conjugate containing 16 1,2-benzanthryl-10-carbamido groups per molecule of protein.

Immunological studies with highly purified conjugates of the polycyclic aromatic hydrocarbons were made in co-operation with Dr. F. S. Cheever of the Harvard Medical School and later on with Drs. Oginsky and Allen at the University of Maryland (14–16). Recent studies with the stilbene conjugates have been made by Dr. H. F. Havas and Miss Janet Fabian at this Institute. Antisera were obtained by injecting rabbits intraperitoneally with amounts of conjugate totaling approximately 500 mg. for the hydrocarbon type and 200 mg. for the stilbene type. Following preliminary determinations by the precipitin procedure, the immunological reactions were studied by micro-Kjeldahl analysis of washed antigen-antibody precipitates with the excellent procedure developed by Dr. Michael Heidelberger (26). Usually, 1 ml. of antiserum plus amounts of antigen in the order of 0.2–3.0 mg. have been adequate to locate the zone of maximum precipitation and to give enough precipitate for accurate nitrogen determinations.

A few of the results of the serological studies are presented in the accompanying tables. Table 1 is a summary of the reactions noted with the polycyclic aromatic hydrocarbon series of conjugates. References 14 and 33 give the structural formulas of the prosthetic groups. The results are expressed in terms of the milligrams of antigen-antibody precipitate obtained per milliliter of antiserum following the addition of 0.2, 0.5, or 1 mg. of test antigen. The first part of the table presents the reactions when the test protein is the same as that used for injection. HSA represents horse serum albumin; bz-HSA-16 and -38 represent horse serum albumin conjugates containing 16 and 38 1,2-benzanthryl groups linked through the 10-position of the molecule. The antisera toward HSA gave satisfactory reactions both with HSA and bz-HSA-38, showing that the latter had retained its protein specificity (lines 1 and 2). Injection of the conjugate bz-HSA-16 likewise produced satisfactory antisera which reacted less strongly with the HSA test antigen (line 3) than with the homologous antigen (line 4), which displayed both prosthetic group activity and protein specificity. The immunizing capacity of the protein component obviously has not been impaired by the introduction of sixteen 1,2-benzanthryl groups. A similar picture is presented in lines 5 and 6 which show, however, that the conjugate containing 38 groups was less effective than the other antigens as an immunizing agent.

Demonstration of prosthetic group activity,

however, requires that the test conjugate contain a different protein from that used in the immunizing antigen as shown in the last part of the table. BovA and USA represent bovine serum albumin and human serum albumin. The formation of antibodies toward 1,2-benzanthryl-10-carbamido horse serum albumin-16 was obviously influenced by the presence of the prosthetic 1,2-benzanthryl group, because a moderately strong serological reaction was noted upon the addition of the bovine albumin conjugate containing fifteen 1,2-benzanthryl groups (line 7). Similarly, human serum albumin with 29 1,2-benzanthryl groups reacted satisfactorily with this antiserum (line 8). Antisera toward bz-HSA-38 reacted with bovine serum albumin conjugates containing 1,2-benzanthracene (bz-BovA-15) and 3,4-benzyprene (BP-BovA-19), as shown in lines 9 and 10. Thus, cross prosthetic group reactivity exists between the hydrocarbons 1,2-benzanthracene and 3,4-benzpyrene. This is not unexpected, since the only factors of importance in this immunological reaction are the size and shape of the nonpolar prosthetic group. Antisera toward bz-USA-29 reacted almost equally well with horse serum albumin conjugates containing sixteen and 38 1,2-benzanthryl groups (lines 11 and 12) and with a bovine serum albumin conjugate with fifteen 1,2-benzanthryl groups (line 13). The amount of antigen-antibody precipitate was less with bovine serum albumin conjugates containing either 1,2,5,6-dibenzanthracene or 3,4-benzpyrene (lines 14 and 15).

Table 2 shows some of the serological reactions in the stilbene series. The structural formulas of the prosthetic groups are given in reference 17. Whenever any line in the table contains no italicized figures, the curve, based upon the amount of the antigen-antibody precipitate, was still rising slightly at the 2-mg. level of antigen/ml of antiserum. It will be noted that good antisera were obtained throughout this series of experiments; for example, horse serum albumin gave antisera which were considerably stronger than those obtained in earlier experiments (line 1, Table 2 versus Table 1). This is partly due to differences between the courses of injection and probably partly due to the rabbits, but predominantly due to bleeding at 5 days after the last injection rather than 10–15 days later. As a result, it is impossible to compare the intensities of the serological reactions recorded in the hydrocarbon series with those in the stilbene series. Antisera toward HSA reacted almost equally well with HSA and with S-HSA-4 which is a horse serum albumin conjugate containing four stilbenyl groups (lines 1 and 2). This observation is of importance, because S-HSA-4 was essentially a control preparation representing serum albumin which had been subjected to the experimental conditions involving alkali and dioxane. Apparently the preparative conditions had little effect on the immunological reactivity and specificity of the albumin. As the amounts of prosthetic group in the conjugate were increased to 55 groups, there were corresponding decreases in the serological reactions of the conjugates with antisera toward HSA (lines 3, 4, and 5). Introduction of 34 stilbenyl groups into horse serum albumin modified the immunizing capacity of the protein to a considerable extent.

TABLE 2

STILBENE SERIES

MG. OF AG.-AB. PPT. PER ML. ANTISERUM

LINE	Antiserum	Antigen	Mg. antigen added 1.0	1.4	2.0
1	HSA	HSA	6.00	*6.24*	5.80
2	HSA	S-HSA-4	6.15	6.48	*6.63*
3	HSA	S-HSA-15	3.10	3.35	*3.80*
4	HSA	S-HSA-34	1.83	2.12	*2.29*
5	HSA	2′-MeS-HSA-55	1.50	1.55	*2.04*
6	S-HSA-34	HSA	2.54	*2.64*	2.63
7	S-HSA-34	S-HSA-4	4.36	4.49	4.65
8	S-HSA-34	S-HSA-15	5.05	5.28	5.58
9	S-HSA-34	S-HSA-34	4.00	4.96	*5.40*
10	S-HSA-34	2′-MeS-HSA-14	4.36	*4.64*	*4.64*
11	S-HSA-34	2′-Mes-HSA-55	2.64	3.37	4.76
12	S-HSA-34	BovA	*0.14*		
13	S-HSA-34	S-BovA-20	2.20	2.52	2.74
14	S-HSA-34	S-BovA-31	2.32	2.47	*2.65*
15	S-HSA-34	2′-MeS-BovA-13	0.45	0.47	*0.53*
16	S-HSA-34	2′-MeS-BovA-37	2.20	2.52	*2.65*
17	S-HSA-34	2′-MeS-BovA-62	2.74	3.44	*4.22*
18	S-HSA-34	2′-MeS-*Azo*-BovA	*0.75*	0.40	
19	2′-MeS-BovA-13	S-HSA-15	*1.15*		1.12
20	2′-MeS-BovA-13	2′-MeS-HSA-55	1.16	1.24	*1.51*
21	2′-MeS-BovA-62	S-HSA-15	*0.54*	0.50	
22	2′-MeS-BovA-62	2′-MeS-HSA-55	0.84	*0.96*	0.93

Prosthetic groups: S = 4-dimethylaminostilbenyl-4′-carbamido; 2′-MeS = 2′-methyl-4-dimethylaminostilbenyl-4′-carbamido. See Table 1 for other symbols.

S-HSA-4 gave a better reaction than native HSA with this antiserum (lines 6 and 7), indicating that even a low number of prosthetic groups may have some influence in the serological reaction involving homologous protein. Again, conjugates with fifteen and 34 groups behaved similarly (lines 8 and 9), indicating that these numbers of groups are within the optimal levels for good prosthetic group reactivity. Conjugates containing 2′-methyl-4-dimethylaminostilbene also reacted well with antisera toward S-HSA-34 (lines 10 and 11).

The reactions demonstrating prosthetic group activity are given in the next part of the table. Antisera toward S-HSA-34 gave a slight reaction when tested with bovine serum albumin (line 12). The conjugates S-BovA-20 and S-BovA-31 reacted well with this antiserum (lines 13 and 14). The bovine serum albumin conjugate containing thir-

teen 2′-MeS groups did not give a very strong reaction (line 15). This probably means that thirteen groups are below the optimum level for the demonstration of satisfactory prosthetic group activity in a test antigen. Conjugates containing 37 and 62 2′-MeS groups gave good reactions with antisera toward S-HSA-34 (lines 16 and 17), thus indicating that the additional methyl group in the stilbene nucleus has little influence in the serological reactions. Tests have also been made with a bovine serum albumin conjugate containing an as yet unknown amount of 2′-MeS component introduced by the azo linkage. Only a small amount of precipitate was produced in this case (line 18). This means either that not enough groups were introduced by the azo linkage to confer strong prosthetic group activity on the protein, or that there is a considerable differentiation between the serological reactions of azo and carbamido conjugates containing the same prosthetic group. The reaction of the albumin with the diazonium chloride of 2′-MeS stilbene was pushed fairly hard, and it would be anticipated that the tyrosine molecules, at least, had been completely substituted. The reaction product was easily fractionated with ammonium sulfate, and the material containing the highest prosthetic group content, as determined colorimetrically, was used in the serological tests.

Although 2′-MeS-BovA-13 was not a good test antigen, it did produce satisfactory antisera (lines 19 and 20). Extensive substitution in the bovine serum albumin to introduce 62 prosthetic groups definitely decreased its immunizing capacity (lines 21 and 22). Tests have also shown that, although S-HSA-4 is a strong immunizing antigen because of its protein component, it does not contain enough stilbenyl groups to show prosthetic group activity. It may be concluded that conjugates with about 30 stilbenyl groups show optimum prosthetic group activity and, therefore, would be expected to be the most satisfactory for immunization against carcinogenesis.

Routine studies were made to determine the amount of antigen in the antigen-antibody precipitates. One-fifth of the alkaline solution of the washed precipitate was analyzed spectrophotometrically to determine the amount of antigen, and a nitrogen determination was made on the remainder of the solution. The molecular ratio of antibody to antigen was found to vary from 3:1 to 1:1. During the course of this work, it was found that exposure of alkaline, aqueous dioxane solutions of the conjugates to light had a greater effect on the intensity of absorption than had been anticipated from earlier work. Consequently in the stilbene series, many of the earlier determinations must be considered incorrect, because undoubtedly there often was more antigen in the precipitate than the absorption measurements indicated. Accurate determinations of the antibody-antigen ratio can be made, however, by observing the proper precautions.

Since, in the serological studies, the test antigens had been used after varying times of exposure to light, a spectrophotometric re-examination was made of all the solutions of the stilbene-type antigens on hand. The majority of the preparations showed a 10 per cent drop in intensity 4 months after dialysis from stock kept under ammonium sulfate solution. Four conjugates, however, showed about a 30 per cent drop in intensity, also with no shift in the wave-length of the maximum. Although there had been no indication that this drop in the intensity of absorption of the stilbene group had any obvious influence on prosthetic group reactivity, comparisons were made between the serological reactions of freshly dialyzed preparations of 2′-MeS-BovA-37 and 2′-MeS-BovA-62 and the same preparations after exposure to light for about 20 hours. The latter samples, which showed a drop in intensity of about 30 per cent, gave from 5 to 15 per cent more precipitate than the former when added to antisera toward 2′-MeS-HSA-14 and 2′-MeS-HSA-55. It appears, therefore, that the chemical changes which take place in the prosthetic group upon exposure to light do not seriously influence its serological reactivity. The amino acid conjugates crystallize in the *trans* form, with a characteristically high intensity of absorption, from solutions in which *trans-cis* equilibrium has been reached. Salting out of the protein conjugate from a solution which had been exposed to light under such conditions that there had been a 30 per cent drop in the intensity of absorption gave material which, after dialysis in the dark, did not exhibit any increase in intensity of absorption. In the latter case, the change appears to have been irreversible.

Possibly some significance should be attached to the observation that greater amounts of precipitate were obtained with the use of the conjugates which had been exposed to light. One interpretation might be that the prosthetic group of the conjugate after injection into the animal undergoes metabolism and that antibodies reflecting the characteristics both of the metabolic product and the unchanged product are elaborated. Based on the above results, one might speculate further that any such metabolite bears some resemblance to that formed upon exposure to light.

It has also been possible to show that such anti-

sera will react with a carcinogen combined with an amino acid or peptide. This was done by the inhibition technic. When an active antiserum toward a protein conjugate is mixed with a water-soluble inhibitor composed of the homologous prosthetic group linked to an amino acid, the antibodies react with the inhibitor but usually no precipitate is produced. Demonstration that a reaction has occurred is shown by the fact that less than the customary amount of precipitate is formed upon subsequent addition of a test antigen composed of the homologous carcinogen-heterologous protein. Occasionally, no precipitate is produced upon addition of the test antigen because all the reactive sites of the antibody molecule have been occupied by the prosthetic group of the inhibitor. Serological reactions in the hydrocarbon series of conjugates were inhibited by compounds made by combining the isocyanates with glycine, E-amino caproic acid, glutamic acid, and glycylglycine (20, 24). Inhibitors having a greater solubility in water were also prepared by enzymatic hydrolysis of the protein conjugates to give products consisting of the prosthetic group combined with a polypeptide or peptone (16). About 25 per cent inhibition of the reaction between antisera toward 2′-MeS-HSA-55 and the test antigen 2′-MeS-BovA-62 was produced by the addition of 2′-MeS-caproic acid.

Tests for carcinogenicity of many of the compounds were made by Drs. Shields Warren and C. E. Dunlap at Harvard University (18) and by Mr. Reed Hankwitz in this Institute. Isocyanates and amino acid conjugates located at the 3- or 10-positions of 1,2-benzanthracene, at the 3-position of 10-methyl- and 9,10-dimethyl-1,2-benzanthracene or at the 5-position of 3,4-benzpyrene have been found to be either inactive or only weakly carcinogenic. On the other hand, 1,2,5,6-dibenzanthryl-9-isocyanate produced fourteen sarcomas in fourteen C3H mice at the site of injection with the first tumor appearing at 92 days and with an average latent period of 111 days (7). This constitutes an exceptional case in which a functional substituent has increased the carcinogenic potency of an active parent compound. The conjugate of this isocyanate with glycine was also an active carcinogen. None of the highly purified protein conjugates has produced tumors under a variety of experimental conditions which have involved either a series of injections or a single large injection of water-soluble material or of the conjugate where the protein component had been deliberately denatured to decrease solubility and to destroy antigenicity. On the other hand, the hydrocarbon prosthetic groups of many of the amino acid and protein conjugates are physiologically active, as has been found in our Institute by Drs. E. U. Green and E. M. Creech (23). These investigators have shown that at a very low concentration, the preparations arrest the development of frogs' eggs and embryos and produce interesting chromosomal abnormalities. The most active conjugate contained 9,10-dimethyl-1,2-benzanthracene as the prosthetic group.

Selection of suitable experimental conditions for studies of the ability of the conjugates to elicit an antibody response capable of preventing carcinogenesis has been difficult. To obtain clear-cut results, one should use an animal which can be readily immunized and which also is susceptible to chemical carcinogenesis. The rabbit fulfills the first requirement and also responds readily to the carcinogenic action of 9,10-dimethyl-1,2-benzanthracene when painted on the ear (8). One experimental difficulty has been the establishment of a dose of carcinogen which will produce tumors in only 50–70 per cent of the rabbits so that any demonstration of immunological protection may be evident. Dr. M. A. Hamilton and I were unable to find the proper dosage and timing of application of this potent carcinogen in the rabbit. Mice are good test animals for the development of tumors due to the polycyclic hydrocarbons, but we have found them difficult to immunize with carcinogen-protein conjugates as judged by serological reactions. Successful experiments will require adequate circulation of antibodies in the areas surrounding the site of painting or injection of carcinogen. Although we are continuing our research with the polycyclic aromatic hydrocarbons, we are interested also in the 4-dimethylaminostilbenes and 2-acetylaminofluorene which are systemic carcinogens. Since these compounds, or again possibly their metabolites, must circulate in the body in order to cause primary tumors in the liver, lungs, ear ducts, kidneys, etc., there may be a better chance of preventing their carcinogenic activity by immunological means. The stilbene conjugates have produced satisfactory antibody responses in rabbits, but, so far, appear to immunize mice only at levels which are well into the lethal range. Attempts are also being made to increase the percentage of tumors caused by these compounds to a higher level than that found by the English investigators, Haddow, Harris, Kon, and Roe (25).

Time alone will tell whether successful immunization against carcinogenesis can be achieved. It is our hope that carefully controlled immunological procedures with well characterized conjugates will enable us to attain that goal.

REFERENCES

1. Butler, G. C.; Harrington, C. R.; and Yuill, M. E. Studies in Synthetic Immunochemistry. 5. Observations on Antisera against Aspirin-Protein Complexes. Biochem. J., **34**:838–45, 1940.
2. Clutton, R. F.; Harrington, C. R.; and Yuill, M. E. Studies in Synthetic Immunochemistry. III. Preparation and Antigenic Properties of Thyroxyl Derivatives of Proteins, and Physiological Effects of Their Antisera. Biochem. J., **32**:1119–32, 1938.
3. Cook, J. W.; Haslewood, G. A. D.; Hewett, C. L.; Hieger, I.; Kennaway, E. L.; and Mayneord, W. V. Chemical Compounds as Carcinogenic Agents. Am. J. Cancer, **29**:219–59, 1937.
4. Coons, A. H.; Creech, H. J.; and Jones, R. N. Immunological Properties of an Antibody Containing a Fluorescent Group. Proc. Soc. Exper. Biol. & Med., **47**:200–202, 1941.
5. Coons, A. H.; Creech, H. J.; Jones, R. N.; and Berliner, E. The Demonstration of Pneumococcal Antigen in Tissues by the Use of Fluorescent Antibody. J. Immunol., **45**:159–70, 1942.
6. Coons, A. H.; Snyder, J. C.; Cheever, F. S.; and Murray, E. S. Localization of Antigen in Tissue Cells. IV. Antigens of Rickettsiae and Mumps Virus. J. Exper. Med., **91**:31–39, 1950.
7. Creech, H. J. Isocyanates of 3,4-Benzpyrene and 10-Methyl-1,2-benzanthracene. J. Am. Chem. Soc., **63**:576–78, 1941.
8. ———. Immunological Properties of Conjugates Prepared from Proteins and Isocyanates of Polynuclear Aromatic Hydrocarbons. Acta Union internat. contre Cancer, **6**:451–55, 1949.
9. Creech, H. J., and Franks, W. R. Compounds Synthesized from Proteins and Carcinogenic Hydrocarbons. Am. J. Cancer, **30**:555–62, 1937.
10. ———. Anthranyl and 1,2,5,6-Dibenzanthranyl Isocyanates. J. Am. Chem. Soc., **60**:127–28, 1938.
11. Creech, H. J., and Jones, R. N. The Conjugation of Horse Serum Albumin with 1,2-Benzanthryl Isocyanates. J. Am. Chem. Soc., **62**:1970–75, 1940.
12. ———. The Conjugation of Horse Serum Albumin with Isocyanates of Certain Polynuclear Aromatic Hydrocarbons. *Ibid.*, **63**:1661–69, 1941.
13. ———. Conjugates Synthesized from Various Proteins and the Isocyanates of Certain Aromatic Polynuclear Hydrocarbons. *Ibid.*, pp. 1670–73.
14. Creech, H. J.; Oginsky, E. L.; and Allen, O. N. Immunological Studies of Hydrocarbon-Protein Conjugates. II. Quantitative Results. Cancer Research, **7**:297–300, 1947.
15. Creech, H. J.; Oginsky, E. L.; and Cheever, F. S. Immunological Studies of Hydrocarbon-Protein Conjugates. I. Precipitin Reactions. *Ibid.*, **7**:290–96. 1947.
16. Creech, H. J.; Oginsky, E. L.; and Tryon, M. Immunological Studies of Hydrocarbon-Protein Conjugates. III. Inhibition Reactions. Cancer Research, **7**:301–4, 1947.
17. Creech, H. J., and Peck, R. M. Conjugates Synthesized from Proteins and the Isocyanates of Certain Systemic Carcinogens. J. Am. Chem. Soc., **74**:463–68, 1952.
18. Dunlap, C. E., and Warren, S. The Carcinogenic Activity of Some New Derivatives of Aromatic Hydrocarbons. II. Compounds Related to 1,2-Benzanthracene. Cancer Research, **6**:454–65, 1946.
19. Fieser, L. F. Carcinogenic Activity, Structure, and Chemical Reactivity of Polynuclear Aromatic Hydrocarbons. Am. J. Cancer, **34**:37–124, 1938.
20. Fieser, L. F., and Creech, H. J. The Conjugation of Amino Acids with Isocyanates of the Anthracene and 1,2-Benzanthracene Series. J. Am. Chem. Soc., **61**:3502–6, 1939.
21. Fieser, L. F.; Fieser, M.; Hershberg, E. B.; Newman, M. S.; Seligman, A. M.; and Shear, M. J. Carcinogenic Activity of the Cholanthrenes and of Other 1,2-Benzanthracene Derivatives. Am. J. Cancer, **29**:260–68, 1937.
22. Franks, W. R., and Creech, H. J. Chemoantigens and Carcinogenesis. Am. J. Cancer, **35**:203–12, 1939.
23. Green, E. U., and Creech, E. M. H. Morphological and Cytological Effects of Carcinogen-Protein Conjugates in the Developing Frog's Egg. Cancer Research, **11**:252, 1951.
24. Green, L. Q., and Creech, H. J. The Conjugation of Peptides with 1,2-Benzanthryl-10-isocyanate. J. Am. Chem. Soc., **68**:2401, 1946.
25. Haddow, A.; Harris, R. J. C.; Kon, G. A. R.; and Roe, E. M. F. The Growth-Inhibitory and Carcinogenic Properties of 4-Aminostilbene and Derivatives. Phil. Trans. Roy. Soc. London, **241**:147–95, 1948.
26. Heidelberger, M., and Kendall, F. E. The Precipitin Reaction between Type III Pneumococcus Polysaccharide and Homologous Antibody. III. A Quantitative Study and a Theory of the Reaction Mechanism. J. Exper. Med., **61**:563–91, 1935.
27. Herriott, R. M. Reactions of Native Proteins with Chemical Reagents. Adv. Protein Chem., **3**:169–225, 1947.
28. Hopkins, S. J., and Wormall, A. Phenyl Isocyanate Protein Compounds and Their Immunological Properties. Biochem. J., **27**:740–53, 1933.
29. Jones, R. N., and Creech, H. J. The Ultraviolet Absorption Spectra of Protein Solutions. J. Optic. Soc. Am., **33**:209–18, 1943.
30. Landsteiner, K. The Specificity of Serological Reactions. Revised edition. Cambridge: Harvard University Press, 1945.
31. Peck, R. M., and Creech, H. J. Isocyanates of Dimethylaminostilbenes and Acetylaminofluorene. J. Am. Chem. Soc., **74**:468–70, 1952.
32. Rodney, G., and Fell, N. Histamine-Protein Complexes: Synthesis and Immunologic Investigation. II. β-(5-Imidazolyl) Ethyl Carbamido Protein. J. Immunol., **47**:251–59, 1943.
33. Smith, W. M., Jr.; Pratt, E. F.; and Creech, H. J. Isocyanates of 9-Methyl- and 9,10-Dimethyl-1,2-Benzanthracene. J. Am. Chem. Soc., **73**:319–22, 1951.

Studies on the Biological Action of Malononitriles*
I. The Effect of Substituted Malononitriles on the Growth of Transplanted Tumors in Mice

EMERY M. GAL,† FUNG-HAAN FUNG, AND DAVID M. GREENBERG

ASSISTED BY HARRIET EZRA AND SHERBURNE F. COOK, JR.

(Department of Biochemistry, School of Medicine, University of California, Berkeley, Calif.)

This laboratory has been engaged for a number of years on a program of cancer chemotherapy research guided mainly by the concept of metabolite antagonism (9) in the selection of compounds to be tested for chemotherapeutic activity. The importance attributed to the components of the nucleic acids in the genesis of neoplasms and the control of cellular functions has, with some measure of success, oriented extensive investigations on experimental tumor chemotherapy. An outstanding example is the work of Kidder and his associates (13) on the growth-inhibiting properties of the purine analog, 8-azaguanine.

Our efforts have been considerably influenced by the work of Hyden and Hartelius (12), who reported that administration of malononitrile ($CH_2[CN]_2$) specifically increased the polynucleotide and protein content of the nerve cells, both in experimental animals and human beings. This effect was described as specific for the neurons. Although this seeming selective action was apparently limited to nervous tissue, while the contrary effect was desired from the point of view of cancer therapy, the results suggested possibilities for undertaking a program for the synthesis of test compounds. It was hoped that the introduction of different substituents into the methylene group of malononitrile would lead to a more generalized and desirable effect without critically altering the reported biological characteristics of the nitriles.

Certain of the results of this work have been published in brief (6–8). A detailed account of the work is presented in this series of papers. The mode of action and the fate of the malononitriles, both in normal and tumor-bearing animals, have been studied.

* This work was aided by grants from the National Cancer Institute, U.S. Public Health Service.

† U.S. Public Health Special Fellow, 1948–50.

Received for publication February 12, 1952.

MATERIALS

Malononitrile, by virtue of its active methylene group, readily condenses with carbonyl compounds to yield dinitriles. The condensation products show different degrees of saturation and can be divided into the following groups:

1. $R\text{-}CH_2CH(CN)_2$
2. $R\text{-}CH\ (CH[CN]_2)_2$
3. $R\text{-}CH{:}C(CN)_2$
4. $R_2\text{-}C{:}C(CN)_2$ (with R_1)
5. $R\text{-}N{:}N\text{-}CH(CN)_2$

With a few exceptions the compounds were prepared in our laboratory according to known procedures (1, 4, 14). The carbonyl compounds used in the syntheses were, for the most part, purchased from Distillation Products Industries. The prepared compounds were checked for purity and the elementary composition of those which were not described in the literature determined by micro-analysis. The choice of substitution was systematically determined by steric relations, as well as by the resemblance to the active groups in some of the known experimental antitumorigenic agents, as, for example, the methoxy groups of colchicine or podophyllin.

Because of the instability of the compounds in alkaline or in acid solutions, they were administered intraperitoneally either in sesame oil (S.O.) or in asymmetrical propylene glycol (P.G.) —the latter because of its toxicity to mice never exceeded a daily dose of 0.05 ml.

To determine the toxicity of the compounds, solutions were injected intraperitoneally into the mice to establish the minimum lethal concentration. With this as the upper level, three dilutions of each compound were injected daily into two normal mice, each for 5 days. The final concentration of the compound chosen for therapy trial was one which caused no mortality and no significant change in weight. The toxicity studies did not reveal any influence of either the sex or the strain of the mouse. The symptoms of toxicity of the malononitriles were much the same as those described for the parent compound by Heymans and Masoin (10).

In the course of these studies, about 500 strain A, 500 C57, and over 1,500 C3H mice were used. The number of animals in a group, whenever possible, was between 10 and 20. The weights of the animals selected before the start of the experiment were between 20 and 25 gm., and the sex ratio was 1:1. At times, young animals were employed in the study, and in each instance this will be specified. The animals received a diet of Purina Laboratory Chow and water ad libitum. They were kept six to eight in a cage, with the sexes separated. The weights of the animals were checked every third day and the

TABLE 1

TOXICITY AND BIOLOGICAL EFFECTS OF MALONONITRILES

Substitution on CH_2-group of malononitrile	Tolerance dose (mg/100 gm)	Vehicle
Group 1:		
γ-Dicyano-methylbutyrate	0.80	P.G.
γ-Dicyano-ethylbutyrate	0.72	P.G.
γ-Dicyano-butyronitrile	0.60	P.G.
Group 2:		
Methylene-bis-	2.0	S.O.
Ethylidene-bis-	3.2	S.O.
Heptylidene-bis-	4.0	S.O.
Group 3:		
Ethoxymethylene-*	0.60	H_2O
Aminomethylene-	20.0	H_2O
Glyceral-†	40.0	H_2O
Citral-†	40.0	H_2O
Tiglal-†	40.0	H_2O
Furanal-	8.0	S.O.
5-Nitrofuranal-*‡	0.80	S.O.
	0.40	P.G.
2-Thenyliden-§	0.80	P.G.
5-Nitro-2-thenyliden-§	0.40	P.G.
Benzal-*‡	1.0	S.O.
	0.80	P.G.
o-Hydroxy-benzal-	6.0	S.O.
m-Hydroxy-benzal-	4.0	S.O.
p-Hydroxy-benzal-*‡	4.0	S.O.
	1.6	P.G.
2,4-Dihydroxy-benzal-	0.80	P.G.
3,5-Dihydroxy-benzal-	1.0	P.G.
o-Methylbenzal-‡	0.80	P.G.
m-Methylbenzal-	1.0	P.G.
p-Methylbenzal-	1.0	P.G.
o-Chlorobenzal-‡	0.40	P.G.
p-Chlorobenzal-*	0.40	P.G.
2-Hydroxy-5-chlorobenzal-	1.0	S.O.
2,4-Dichloro-benzal-	0.80	P.G.
2,6-Dichloro-benzal-	0.80	P.G.
o-Nitrobenzal-‡	1.0	S.O.
m-Nitrobenzal-	1.2	S.O.
p-Nitrobenzal-*	1.8	S.O.
	0.8	P.G.
2,4-Dinitro-benzal-	1.6	P.G.
2,6-Dinitro-benzal-	1.6	P.G.
2-Chloro-5-nitrobenzal-‡	1.0	P.G.
p-Methoxy-benzal-	0.8	P.G.
2,4-Dimethoxy-benzal-	0.8	P.G.
2,5-Dimethoxy-benzal-	1.0	P.G.
3,4-Dimethoxy-benzal-	1.2	P.G.
3,4,5-Trihydroxy-benzal-†	4.8	S.O
	1.6	P.G.
3,4,5-Trimethoxy-benzal-†	8.0	S.O.
3-Methoxy-4-hydroxybenzal-	2.0	S.O.
Piperonal-*§	0.4	S.O.
Cinammal-	1.2	S.O.
p-Acetylamino-benzal-	0.60	S.O.
p-Dimethylamino-benzal-	0.80	S.O.
p-Diethylamino-benzal-	1.2	S.O.
1-Ethoxy-2-naphthal-	0.72	P.G.
9-Anthral-	0.80	P.G.
Chinchoninal-	1.00	S.O.
Group 4:		
Methyl-benzylidene-	0.80	P.G.
Methyl-(*p*-chlorophenyl)ethylidene-	0.72	P.G.
1-Methyl-cyclopropyl-ethylidene-§	1.92	P.G.
1-Methyl-(*p*-flurophenyl)ethylidene-	1.20	P.G.
1-Methyl-(*p*-methylphenyl)ethylidene-	1.40	P.G.
1-Methyl-(*p*-nitrophenyl)ethylidene-*	0.80	P.G.
1-Propyl-(phenyl)ethylidene-	1.40	P.G.
1-Methyl-(furanyl)ethylidene-*	1.25	P.G.
1-Methyl-(thienyl)ethylidene-§	1.0	P.G.
Isatyl-	1.4	S.O.
	1.0	P.G.

TABLE 1—*Continued*

Substitution on CH_2-group of malononitrile	Tolerance dose (mg/100 gm)	Vehicle
Group 5:		
Phenylazo-‖	1.6	S.O.
p-Dimethylamino-phenylazo-†‖	12.0	S.O.
p-Nitrophenylazo-‖	1.80	S.O.
p-Chlorophenylazo-‖	1.0	S.O.
Miscellaneous:		
1,3-Dimethyl-cyclobutane 2,2,4,4-tetranitrile	4.0	S.O.
2-Cyano-1,4-endomethylene cyclohexene‡ (Δ4,5)	0.4	P.G.
Fumaronitrile§	3.0	S.O.

* Induced tumor-growth inhibition.
† First synthesized in our laboratory.
‡ Irritant of skin and mucous membranes.
§ Obtained from Monsanto Chemical Co., through the courtesy of Drs. F. C. Meyer and A. H. Schlesinger.
‖ Slight tumor growth-promoting effect.
P.G. = Asym. propylene glycol.
S.O. = Sesame oil.

diameters of the tumors every fifth day. No caliper measurements were taken on the Gardner lymphosarcoma-bearing animals. At the end of the experiment, which usually took 2-3 weeks, the animals were sacrificed, the tumors carefully removed, weighed, and photographed, and the results recorded. Some of the tumors were preserved for histological examination.[1] The mouse tumors employed were Sarcoma 37, 6C3H-ED (lymphosarcoma), the carcinomas LCS-A, C3H-S,[2] C57-E 0771 and C57-C1498 (Bar Harbor myeloid leukemia).

The transplantation of the tumors was carried out by injecting, by means of a trocar, uniform small pieces of non-necrotic tumor tissue, obtained under aseptic conditions, into the axillary region of one side. In transplanting, donor tumors grown for the same time period were used, and the pieces from each tumor were evenly distributed among each of the experimental groups. Two to 6 days were allowed for the tumors to take. After 6 days, all the animals without palpable tumors were eliminated, and those with tumors made up the final groups for the injection of the compounds. In the grouping of these animals, the range of size of the tumors and the weight of the animals were kept as uniform as feasible. In some experiments the food intake was also checked.

Statistical analysis was made only on those animals that survived or died within a day or two before the termination of the experiment. The standard deviation (σ), standard error of the mean (Σ[M]) and standard error of difference of the means (Σ[D]) were calculated. The calculation of the t values for certain curves were at times a necessity in order to appraise the significance of the difference.

Because of the great number of the compounds tested detailed experimental data will be restricted to those compounds which showed a marked effect on the tumor growth.

The list of compounds tested, the tolerated dosage, and certain physiological effects are reported in Table 1.

[1] We gratefully acknowledge our great indebtedness to Dr. Elizabeth Lowenhaupt for histological examination of representative tumor specimens.

[2] This adenocarcinoma, kindly furnished us by Dr. M. Shimkin, was started on April 26, 1948, as a transplant from a spontaneous mammary carcinoma arising in a female C3H mouse of the Andervont subline. It is now (March 24, 1952) in the 83d generation of transplantation. The other tumors employed are well known and widely used and do not require special reference citations.

RESULTS AND DISCUSSION

The first experiment was designed to test whether the effect of the malononitriles, if any, was due to the release of cyanide or of some of the most probable intermediates of their catabolism on the basis of their relative toxicity. For this test aqueous solutions of sodium cyanide, malononitrile, sodium thiocyanate, acetonitrile, cyanacetamide, malonamide, and malonic acid were injected into both LCS-A- and C3H-S-bearing mice. No effect on the tumor growth was observed.

Our finding regarding cyanide itself agrees with Bullock's (3) observation on the growth of the Ehrlich sarcoma, on which it also had no effect. Compounds of groups 1, 2, and 4 showed no effect on tumor growth either, and those of group 5, interestingly enough, showed a slight acceleration of growth rather than retardation.

At a very early stage of our investigation we noticed that ethoxymethylenemalononitrile (C_2H_5-O·CH:C[CN]$_2$) retarded the growth of carcinomatous tissue, particularly that of the LCS and to a lesser extent of C3H-S. The results are given in Table 2.

In experiments 1, 8, and 10, the food intake was checked every second day, and no difference from the controls was found. The compound was administered in freshly prepared aqueous solution. When the compound was given in a daily dose of 50 μg., as in experiment 4, the effect was not too appreciable. A slight loss of body weight was noticeable only in experiment 2, but, in general, the administration did not influence the body weight, nor had it any effect on the mortality rate. The average loss of body weight was computed after the tumor weight had been subtracted. The figures were obtained by means of the following expression:

$$\Delta \text{ (change in body wt.)} = \frac{(\text{[final wt.} - \text{tumor wt.]} - \text{initial body wt.})}{\text{number of animals analyzable}}.$$

This was abbreviated to read

$$\Delta \text{ Wt.} = \frac{(fw - tw) - iw}{N}.$$

However, we have found that the LCS tumor shows very irregular takes and that very often, even after it has taken, some tumors of the control group showed regression during the later course of the experiments. Furthermore, strain A mice, in our experience, proved to be less sturdy than either C3H or C57 mice. These arguments, however, do not apply to experiment 10, where, although the compound was injected for over a week after transplantation had taken place, the analysis still showed a probable effect.

Interesting is the observation, drawn from the caliper measurements, that the compound seemed to be most effective during the mid-course of the treatment and that the effect tended to taper off, as if the rapidly growing tissue had developed a tolerance to the nitrile and could not be affected any further.

We have also attempted, in view of the relatively nontoxic character of the compound, to inject it alternately with 8-azaguanine into strain A mice bearing the LCS tumor. This combined treatment, when statistically analyzed, did not show any greater effect on the retardation of growth of the LCS carcinoma than did 8-azaguanine itself.

TABLE 2

EFFECT OF ETHOXYMETHYLENEMALONONITRILE ON TUMOR GROWTH

Exp. no.	Tumor	No. animals*	Daily† dose (mg/100 gm)	Days between transplant and treatment	Days of treatment	Mean tumor wt. (gm.)	σ	Σ(M)	Σ(D)	Δ Wt. (gm.)	Regressions*	Mortality
1	LCS-	10(7)		6	10	0.514	0.220	0.083	0.106	+0.6	2	1
	Control	10(7)	400			0.955	0.167	0.067		+0.2		3
8	LCS	15(15)		8	14	1.558	0.630	0.160	0.333	+0.7		1
	Control	15(9)	400			3.568	1.130	0.292		+0.8		0
4	LCS	20(19)		8	10	0.800	0.425	0.098	0.146	+0.3		0
	Control	20(20)	200			1.056	0.503	0.112		+0.5		6
2	S37	10(6)		6	9	1.164	0.190	0.077	0.103	−0.1		4
	Control	10(6)	400			1.083	0.165	0.068		+1.4	1	3
3	6C34-ED	10(6)	800	6	9	1.008	0.168	0.068	0.082	+3.3		4
	Control	10(6)				1.147	0.114	0.046		+4.3		4
10	C3H-S	13(9)	600	8	15	2.834	0.972	0.323	0.473	+3.2	2	2
	Control	13(12)				4.147	1.200	0.346		+3.2		1

* No. of animals analyzed statistically are in parentheses. Regressions are not included in the analysis.
† Given in 0.1 ml. water and expressed in mg/100 gm body weight.

As a consequence of the above-mentioned difficulties, C3H and C57 mice have been used almost exclusively in the rest of our experiments.

Since, as Table 1 shows, we did not find any other nitrile of the aliphatic series to possess any growth-retarding property, we turned our attention to the aromatic and heterocyclic substituted malononitriles. These malononitriles were screened on C3H-S-bearing mice, as this tumor showed excellent takes and is known to be among the least resistant to chemotherapeutic agents. Most of the malononitriles had no effect on the growth of the C3H-S tumor, so they were not expected to have any effect on other tumors; and also, because of the necessity of economy of effort, they were not further screened on other tumors. While this approach seemed to be the most practical, the possibility exists that some of the nitriles might, perhaps, have possessed growth-retarding effect on other tumors.

The results of substitutions on the benzene ring of the aromatic malononitriles were extremely interesting. Although the ortho-substituted malononitriles had no effect on the tumor growth, they were found to be powerful irritants to skin and mucous membranes. Meta-substitution did not lend any particular biological effect to the compounds. Para-substitution of the aromatic malononitriles introduced none of the irritating properties of ortho-substitution. In their action on the retardation of tumor growth, however, they ranged from none to a significant retarding effect. One might express the effect of para-substitution somewhat loosely in the following order of magnitude:

$$NO_2 > HO > Cl > CH_3O > CH_3.$$

TABLE 3

RETARDING EFFECT OF *p*-NITROBENZALMALONONITRILE ON TUMOR GROWTH

Exp. no.	Tumor	No. animals*	Daily dose (mg/100 gm)	Days between transplant and treatment	Days of treatment	Mean tumor wt. (gm.)	σ	$\Sigma(M)$	$\Sigma(D)$	ΔWt.	Regressions*	Mortality
49	LCS	13(10)	1.80	6	21	0.172	0.266	0.084	0.267	+4.2		3
	Control	13(8)	0.5 ml. S.O.			0.826	0.720	0.255		+5.4	4	1
51	S37	15(7)	1.50	7	14	1.014	0.800	0.280	0.444	−4.4		10
	Control	15(12)	0.05 ml. S.O.			1.990	1.067	0.345		−5.2		6
53	6C3H-ED	20	1.00	4	15	No effect on the survival				−0.5	2	†
	Control	20	0.05 ml. P.G.							−0.2		†
55	Myeloid Leukemia	15	0.80									
		15	0.05 ml. P.G.	1	11	No effect on the survival				+2.2		†
	Control									+1.7		†
50‡	C3H-S	15(7)	1.75	7	11	0.173	0.129	0.053	0.401	+0.20	2	6
	Control	15(12)	0.05 ml. S.O.			2.100	1.390	0.398		+0.24		3
52	C3H-S	20(14)	1.00	5	14	1.530	0.998	0.266	0.526	−3.0	1	5
	Control	20(15)	0.05 S.O.			3.280	2.400	0.454		−0.5		5
61	C3H-S	20(18)	0.80	7	14	1.900	0.940	0.220	0.280	+0.7	1	1
	Control	20(18)	0.1 ml. S.O.			3.000	0.870	0.205		+1.4		2
64	C3H-S	20(14)	0.80	8	14	2.100	0.825	0.212	0.381	+1.8		6
	Control	20(15)	0.2 ml. S.O.			3.320	1.230	0.317				6
62	C3H-S	15(15)	0.80§	2	20	2.450	0.725	0.187	0.399	−0.8		1
	Control	20(19)	0.2 ml. S.O.			3.180	1.500	0.343		−0.8		1
68	C3H-S	25(7)	4.00	7	12	1.630	0.670	0.252	0.393	−1.9	5	13
	Control	20(16)	0.4 ml. S.O. +0.5 ml. $S_2O_3^{--}$			3.300	1.210	0.302		+2.0		4
54	C3H-S	11(3)	0.80	2	21	1.600	0.790	0.430	0.838	−1.5	4	4
	Control	11(11)	0.05 ml. P.G.			2.577	2.400	0.720		−0.6		0
67	C3H-S	20(12)	0.80	4	16	2.700	0.580	0.163	0.387	−4.2		8
	Control	20(15)	0.05 ml. P.G.			4.600	1.370	0.352		−3.3		5
59	E 0771-C57	20(18)	0.40	7	8	1.800	0.895	0.210	0.292	−5.8		7
	Control	33(33)	0.1 ml. S.O.			3.000	1.130	0.203		−5.8		6‖
57	E 0771-C57	12(4)	0.60	11	12	0.900	0.665	0.332	0.424	−2.4		8
	Control	12(7)	0.05 ml. P.G.			1.770	0.755	0.283		−1.3		5

* No. of animals analyzed statistically are in parentheses. Regressions are not included.
† Run until all controls died.
‡ Young animals.
§ Injected every other day.
‖ Died the day exp. terminated.

It was not possible to ascertain any such approximate order of activation with di- or tri-substitutions on the benzene ring.

Table 3 summarizes the experiments with *p*-nitrobenzalmalononitrile (PNBMN) (NO_2-C$_6$H$_4$-CH:C[CN]$_2$) carried out after it was found in experiments 49 and 50 that this compound slowed down the growth of the carcinoma.

The mortality was high, even though in the treated animals there was no loss of body weight. This was attributed to the fact that, in experiment 50, young animals were used. In the experiments that followed, the daily dose of the nitrile was reduced, and older animals with an average initial body weight of 25 gm. were employed. The results were encouraging with respect to the retardation of the tumor growth, the body weight changes, and the mortality, in contrast to those in the control animals. All attempts failed in an effort to increase the effectiveness of the compound either by change of the vehicle from sesame oil to asymmetrical-propylene glycol or by administering higher doses of the nitrile. Administration in propylene glycol increased the mortality rate and made use of smaller doses of the compound imperative at the expense of its effectiveness.

When PNBMN was administered every other day, as in experiment 62, no growth-retarding effect was observed. In experiment 68 (Table 3) high doses of PNBMN were given with 4 times the equivalent doses of sodium thiosulfate. It was expected that, as in the nitrile detoxications described by Hunt (11), the effect of cyanide, if any, in causing the high mortality could be reduced by the administration of sodium thiosulfate. However, it did not give any protection, and, on the contrary, the mortality rate was increased. PNBMN had no effect on either the 6C3H-ED (Gardner lymphosarcoma) or on the myeloid leukemia C1498 (Bar Harbor). Its effect on E 0771-C57 is questionable. In Table 3 it is shown that, out of a total of 495 animals, the mortality was 42.8 per cent in the treated animals against 31.5 per cent for the controls. None of the other aromatic malononitriles showed nearly so strong an effect as PNBMN.

We were led to believe that the orientation of the nitrile group para to the malononitrile might be a requirement for its action. Accordingly, the investigation was extended to the oxy- and thioheterocyclic malononitriles, and it was found that 5-nitrofuranalmalononitrile (5-NFMN) (NO_2-C$_4$H$_2$O-CH:C[CN]$_2$) and also 5-nitro-2-thenyliden-malononitrile (5-NTMN) caused a retardation of tumor growth. It is to be noted that the 5-nitro substitution on the above heterocyclic rings is indeed essentially equivalent to the para position with respect to the malononitrile. Our results with 5-NFMN are summarized in Table 4. The results on 447 animals are totaled and analyzed in Table 4. The mortality of the treated animals reached 59.5 per cent, while that of the controls was 36.3 per cent.

When the volume measurements are plotted against time, they appeared to indicate a trend which implied that the tumor growth was slowed down but the tumors became somewhat resistant to the treatment.

The rate of growth (r) can be calculated from the volume (V) according to the equation $\sqrt[3]{\frac{V}{0.523}} = r(t - t_0)$, which can be arrived at from the two equations $d = r(t - t_0)$ and $V = 0.523d^3$ described by Schrek (15), when d is the mean diameter, t is the period of treatment, and t_0 the latent period.

Plots of the measured volumes of the tumors against time indicate the changing trends in the rates of tumor growth with the progress of the treatment plotted. Analysis of the rate of growth of the animals treated with 5-NFMN and of the controls gave r values of 0.78 and 1.23 mm., respectively, at the termination of the experiment. The mean diameter, d, was between 0.3 and 0.4 cm. at the time of the beginning of treatment for each group.

As an example of the growth retardation that is obtained, the tumors of experiment 72 have been photographed, and the difference in size is shown in Figure 1 between the controls (1) and the 5-NFMN–treated (2). Illustrative and more significant, perhaps, is an analysis of the distribution of the tumor weights of the treated animals versus the controls. In Chart 1 there is plotted the percentage distribution of the size of 308 individual C3H-S tumors; 58 from 5-NFMN–treated, 90 from PNBMN-treated, and 160 from control animals.

At first glance one would be inclined to ascribe the effect of the malononitriles to their nitrile group or to the action of cyanide resulting therefrom. However, the effect of these compounds on the tumors can hardly be attributed to cyanide release, because the injection of equivalent amounts of cyanide had no effect on the tumor growth. Favorable to this deduction is the observation that no appreciable thiocyanate appears in the urine after 5-NFMN and PNBMN treatment, while a

considerable amount of thiocyanate appears after the administration of malononitrile or of other substituted benzalmalononitriles, such as the benzalmalononitrile itself. Furthemore, the toxicity of malononitriles or of nitriles that yield a high percentage of thiocyanate can be counteracted by thiosulfate, whereas the PNBMN and 5-NFMN–treated animals responded very poorly, if at all, to thiosulfate administration.

The nitrile group itself in these compounds is not the causative factor, as 90 per cent of the malononitriles tested exhibited no effect on tumor growth.

We believe that one of the most important features of modern chemotherapeutic research on cancer ought to be the correlation of the growth-retarding effect of a compound with its structural make-up and the establishment of its interplay on the chemistry of cellular processes. It was such a consideration that led to the systematic, although unsuccessful, exploration of the effect of substitution on the malononitriles. In the three most active compounds, substitution by the nitro group in the para position was established as being desirable. The effect, however, was not due solely to the para nitro position, as none of the parent aldehydes of the reactive malononitriles exhibited any retarding effect on tumor growth, although it is known from the work of Boyland and Mawson (2) that certain aldehydes do inhibit the growth of Crocker Sarcoma 180 in mice. The difference in response is no doubt due to the dissimilarity of the tumors. Furthermore, if the malononitrile moiety of the molecule was replaced by thiosemicarbazide, the growth-retarding action was, interestingly enough, replaced by that of a growth-accelerating effect. That the specificity of action seems to be related

TABLE 4

RETARDING EFFECT OF 5-NITROFURFURALMALONONITRILE ON TUMOR GROWTH

Exp. no.	Tumor	No. animals*	Daily dose (mg/100 gm)	Days between transplant and treatment	Days of treatment	Mean tumor wt. (gm.)	σ	$\Sigma(M)$	$\Sigma(D)$	Δ Wt. (gm.)	Regressions*	Mortality
61	C3H-S	20(15)	0.60	6	14	0.890	0.538	0.138		−3.0		9
	Control	20(18)	0.1 ml. S.O.			3.000	0.870	0.205	0.249	+1.4		2
72	C3H-S	30(18)	0.60	2	14	0.900	0.470	0.113	0.223	−2.5		12
	Control	30(25)	0.1 ml. S.O.			3.200	0.965	0.193		+1.3		5
64	C3H-S	20(14)	0.20	8	12	2.239	0.565	0.151	0.347	−2.4		6
	Control	20(15)	0.1 ml. S.O.			3.320	1.230	0.317		0.0		6
67	C3H-S	20(11)	0.20	3	14	0.980	0.352	0.105	0.160	−3.3	1	8
	Control	15(14)	0.05 ml. P.G.			1.650	0.447	0.120		+0.1	1	0
55	Myeloid Leukemia	15	0.30	1	11	No effect on the survival				−0.4		†
	Control	15	0.05 ml. P.G.							+1.0		
75	6C3H-ED	55	0.30	2	25	No effect on the survival				−1.5		†
	Control	55	0.05 ml. P.G.							+2.6		†
59	E 0771-C57	20(16)	0.40	7	8	2.520	1.090	0.271	0.338	−3.1		4
	Control	33	0.1 ml. S.O.			3.000	1.130	0.203		−5.8		6‡
69	E 0771-C57	20(9)	0.32	2	14	1.500	0.520	0.173	0.361	−1.1	2	9
	Control	30(16)	0.1 ml. S.O.			2.220	0.495	0.136		−0.4		7
63	E 0771-C57	19(7)	0.32	2	21	1.577	0.620	0.236	0.334	−1.9	3	9
	Control	30(16)	0.1 ml. S.O.			2.220	0.950	0.237		−2.7	6	8

* No. of animals analyzed statistically are in parentheses. Regressions are not included in the analysis.

† Run until all controls died.

‡ Died at the termination day of the experiment.

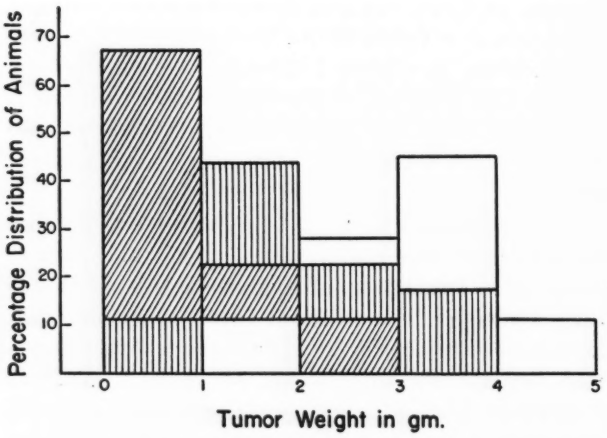


CHART 1.—The effect of ▨ 5-NFMN and ▥ PNBMN on the percentage distribution of tumor weights in C3H-S bearing mice. (□ Control.)

uniquely to a set structural pattern is shown in Table 5.

The great toxicity of the compounds at their most effective levels of administration and the frequent loss of weight by the animals, as a consequence, raise the question whether the growth retardation is not merely a result of the consequences of anorexia and malnutrition. If this is true, then, for all intents and purposes, the growth retardation of the tumors should tend to become a function of the loss of weight of the experimental animals. This did not appear to be the case, as Table 6 demonstrates.

It is apparent from Table 6 that several aromatic malononitriles, such as the *p*-acetylamino- and the 2,4-dinitrobenzal derivatives, which were administered in far higher doses than either PNBMN or 5-NFMN, did not affect the tumor growth, yet, nevertheless, caused a loss of body weight. In experiments where the food intake was controlled, the weight changes of the treated animals did not show any significant difference from that of the controls, when totaled for the entire duration of the treatment.

We cannot sufficiently emphasize that, although some of the malononitriles slowed down the rate of growth, none of them produced complete arrest of growth nor regression of the tumors. Statistical analysis of the material helped to establish whether the differences between the mean tumor weights of the treated and of the control animals belonged to the same population or not. As the $\Sigma(D)$ values in the tables attest, many of our experimental observations are statistically significant, some of them are probably significant, and, again, many are not significant. It is now clear to us that any retardation of growth caused by a chemical that is below 50 per cent of the control growth, or is above 50 per cent, but is statistically attributable to chance, should be considered negative.

In this sense we may state that PNBMN,

TABLE 5

SUMMARY OF THE EFFECT OF THE ACTIVE MALONONITRILES AND THEIR CONGENERS ON TRANSPLANTED TUMORS

Compound	Daily dose (mg/100 gm)	Vehicle*	Tumors* LCS-A	S37-A	C3H-S	6C3H-ED	C 1493 myeloid leukemia	E 0771
Malononitrile	0.16	water	−	−	−	−	−	−
Benzalmalononitrile	1.00	S.O.						
	0.80	P.G.	±	−	±	−	−	−
p-Nitrobenzalmalononitrile	1.80	S.O.						
	1.00	P.G.	+	±	++	−	−	+
p-Nitrobenzaldehyde	0.80	P.G.	−	−	−	−	−	−
p-Nitrobenzalthiosemicarbazone	0.80	P.G.	−	−	×	−		×
Furanalmalononitrile	4.00	S.O.						
	0.80	P.G.	±	−	−	−	−	−
5-Nitrofuranal	0.80	S.O.						
	0.32	P.G.			±	−		−
5-Nitrofurfurylcyanoacetic acid	1.20	P.G.			−	−		−
5-Nitrofuranalsemicarbazone (Furacin)	2.00	P.G.				−		
5-Nitrofuranalthiosemicarbazone	0.40	P.G.			×			
5-Nitrofuranalmalononitrile	0.60	S.O.						
	0.30	P.G.			++	−	−	+
2-Thenylidenmalononitrile	0.80	P.G.			−	−	−	−
5-Nitrothenylidenmalononitrile	0.40	P.G.			++	−	−	+

* Designations represent values of $\frac{m_1 - m_2}{\Sigma(D)}$ >2.0 = ±; >2.5 = +; >3.0 = ++.

Growth promoting effect = ×; no effect = −.

† S.O. = sesame oil; P.G. = asym. propylene glycol.

TABLE 6

THE EFFECT OF ADMINISTRATION OF MALONONITRILES ON BODY WEIGHT OF ANIMALS

Exp. No.	Groups	Statistically analyzable no. of animals*	Av. initial body wt. (gm.)	Av. Δ body wt. (gm.)	Av. tumor wt. (gm.)
61	Tumor C3H-S:				
	p-acetylaminobenzal	16	23.6	−2.9	2.53
	2,4-nitrobenzal	19	26.1	−0.1	2.40
	p-nitrobenzal	18	24.7	+0.7	1.90
	5-nitrofuranal	15	23.2	−3.0	0.89
	Control	18	24.3	+1.4	3.00
59	Tumor C57-E 0771:				
	Control	33	25.9	−5.8	3.00
	p-nitrobenzal	18	24.8	−5.8	1.80
	p-acetylaminobenzal	19	25.5	−5.1	2.70
	5-nitrofuranal	16	25.7	−3.1	2.52
50	Tumor C3H-S:				
	Control	12	20.0	+0.20	2.10
	p-nitrobenzal	7	17.8	+0.24	0.17
51	Tumor S37-A:				
	Control	12	29.1	−4.4	1.99
	p-nitrobenzal	7	26.3	−5.2	1.01

* Only animals that survived for duration of the experiment.

5-NFMN, and 5-NTMN have a statistically significant growth-retarding effect on C3H-S, a probable effect on E 0771-C57, and none on S 37, myeloid leukemia C1498, and 6C3H-ED. This latter tumor was also used to test the effect of the compounds on the survival time of the animals. None of the compounds showed a significant effect on the survival of the animals. We would like to mention that we have also tested the 5-nitrofuranalsemicarbazone (Furacin) and could not confirm the reported beneficial effect (5) on the survival of 6C3H-ED bearing mice. We have also attempted combined treatments employing colchicine, podophyllin, aminopterin, and desoxypyridoxine in combination with the effective malononitriles. We hoped for a reciprocal enhancing effect, but the results showed that the combined effect on the tumor growth was not additive.

It is hoped that the systematic studies on the relation of structure of the chemical agent to the growth retardation of tumors now in progress in our laboratory may shed some interesting information on this difficult and time-consuming problem of cancer chemotherapy.

SUMMARY

1. Systematic studies on the different substituted malononitriles showed that ethoxymethylenemalononitrile of the aliphatic series had some effect on the retardation of growth of carcinoma. *p*-Nitrobenzal-, 5-nitrofuranal-, and 5-nitro-2-thenylidenmalononitriles retarded the growth of the transplantable carcinomas, C3H-S and E 0771 to an even greater degree.

2. This effect is attributable to the structural properties of the particular compounds rather than to the release of cyanide at different rates. Neither sodium cyanide nor malononitrile itself showed any growth retardation on the tumors tested.

3. The significance of the observations was statistically appraised and the results discussed.

REFERENCES

1. Baddiley, D.; Lythgoe, B.; and Todd, A. R. Experiments on the Synthesis of Purine Nucleotides. Part II. A New and Convenient Synthesis of Adenine. J. Chem. Soc., pp. 386–87, 1943.
2. Boyland, E., and Mawson, E. H. Experiments on the Chemotherapy of Cancer. The Effect of Aldehydes and Glucosides. Biochem. J., **32**:1982–87, 1938.
3. Bullock, F. D. The Influence of Sodium Cyanide upon Ehrlich Sarcoma. Proc. Soc. Exper. Biol. & Med., **14**:122, 1916.
4. Corson, B. B., and Stoughton, R. W. Reactions of α, β-Unsaturated Nitriles. J. Am. Chem. Soc., **50**:2825–37, 1928.
5. Friedgood, C. E., and Green, M. N. The Effect of Nitrofurazone on Growth of Fibrosarcoma in Mice. Cancer Research, **10**:613–15, 1950.
6. Gal, E. M.; Ezra, H.; Fung, F. H.; and Greenberg, D. M. The Effect of Substituted Malononitriles on the Growth of Transplant Tumors in Mice. Cancer Research, **11**:249–50, 1951.
7. Gal, E. M., and Greenberg, D. M. Growth-Retarding Effect of Substituted Malononitriles on Transplant Tumors in Mice. J. Am. Chem. Soc., **73**:502, 1951.
8. Greenberg, D. M.; Irish, A. J.; and Gal, E. M. Effect of Metabolite Analogs on Growth of Transplantable Tumors. Cancer Research, **10**:221–22, 1950.
9. Greenberg, D. M., and Schulman, M. P. Application of "Metabolite Antagonism" to Cancer Research. Science, **106**:271–72, 1947.
10. Heymans, J. F., and Masoin, P. Étude physiologique sur les dinitriles normaux. Arch. de Pharmacodynamie et de Thérap., iii: 77–172, 1897.
11. Hunt, R. Zur Kenntnis der Toxikologie einiger Nitrile und deren Antidote. Arch. Internat. de Pharmacodynamie, **12**:447–79, 1904.
12. Hyden, H., and Hartelius, H. Stimulation of the Nucleoprotein Production in the Nerve Cells by Malononitrile and Its Effect on Psychic Functions in Mental Disorders. Arch. Psych. & Neurol. (Suppl.), XLVIII:1–117, 1948.
13. Kidder, G. W.; Dewey, V. C.; and Parks, R. E., Jr. Purine Metabolism in Tetrahymena and Its Relatior to Malignant Cells in Mice. Science, **109**:511–14, 1949.
14. Mowry, D. T. The Knoevenagel Condensation of Acylalkylketones with Malononitrile. J. Am. Chem. Soc., **67**:1050–51, 1945.
15. Schrek, R. A Quantitative Study of the Growth of the Walker Rat Tumor and the Flexner-Jobling Rat Carcinoma. Am. J. Cancer, **24**:807–22, 1935.

Fig. 1.—Photograph showing average size of tumors (C3H-S) in controls (1) contrasted to 5-nitrofuranalmalononitrile treated (2) animals (Experiment No. 72).

1
2

Addendum
Substituted Malononitriles in Neoplastic Diseases in Man

N. L. Petrakis, H. R. Bierman, and M. B. Shimkin

(Laboratory of Experimental Oncology, National Cancer Institute, National Institutes of Health, Public Health Service, Federal Security Agency and the Division of Medicine, University of California School of Medicine, San Francisco, Calif.)

Gal, Fung, and Greenberg (1) showed that certain substituted malononitriles partially inhibited the growth of some transplantable tumors in mice. Clinical trial of two chemicals from their series was therefore undertaken.

Nine patients, as given in Table 1, were selected. All had advanced neoplastic disease, substantiated by relevant biopsy. Periodic clinical examinations, including measurement of accessible tumors, were performed. Weekly examinations were carried out on the blood and urine, as well as chemical determinations of the blood for albumin, globulin, nonprotein nitrogen and bilirubin.

p-Nitrobenzalmalononitrile was dispersed as a 5 per cent suspension in sesame oil, and 5-nitrofuranalmalononitrile was dispersed as a 10 per cent suspension in propylene glycol. The chemicals were injected intramuscularly, once a day in 7 patients and every other day in one patient; one patient received a single dose only. Thus, daily doses of *p*-nitrobenzalmalononitrile were up to 15 mg/kg body weight; and of 5-nitrofuranalmalononitrile, up to 40 mg/ kg body weight.

All patients complained of considerable pain at the site of injection, but no severe local reactions were observed. There were no acute effects upon pulse, respiration, temperature, blood pressure, or electrocardiographic patterns. No significant or consistent alterations in the blood cell counts, urine, or chemical constituents of the blood were detected.

Blood levels of thiocyanate were studied in five patients. Following a single injection of 0.7 gm. of *p*-nitrobenzalmalononitrile, the blood thiocyanate rose from 0.1 to 0.8 mg/100 cc within 24 hours in one patient; in the second patient, the blood thiocyanate level rose steadily from 0.6 to 3.6 mg/100 cc during a course of 5.35 gm. given in 13 days. No significant or consistent rises were seen in three patients who received 1.5, 1.8, and 2.0 gm. of 5-nitrofuranalmalononitrile, respectively.

In none of the patients was there an observable effect upon the primary tumor or its metastases. No toxic effects were seen, and none of the patients derived subjective or objective clinical improvement. All patients died after an expected course of illness. Autopsy examination was available on eight patients, from 1 day to 7 months following completion of injections. No unusual histologic features were present either in the tumors or in the tissues of the body.

It is concluded that *p*-nitrobenzal- and 5-nitrofuranalmalononitrile, at the doses, length of courses, route, and vehicles employed had no effect upon the specific neoplasms observed in nine patients, and that no toxic effects were elicited.

TABLE 1

SUBSTITUTED MALONONITRILES IN PATIENTS WITH CANCER

Case no.	Sex	Age	Diagnosis	Dose/day (gm.)	Days used no.	Total dose gm.
			p-nitrobenzalmalononitrile			
1	M	39	Carcinoma, nasopharynx	0.7	1	0.7
2	M	62	Carcinoma, lung	0.03–0.15	18	1.61
3	M	19	Osteogenic sarcoma	0.1 –0.9	13	5.35
			5-nitrofuranalmalononitrile			
4	F	47	Carcinoma, antrum	0.1	10	1.0
				2.0	1	2.0
5	M	49	Carcinoma, nasopharynx	0.1 –0.2	10	1.1
				0.3 –1.0	9	7.0
6	M	40	Carcinoma, nasopharynx	0.1 –0.9	10	5.4
7	M	25	Carcinoma, testis	0.1 –0.5	5	1.5
8	M	20	Osteogenic sarcoma	0.3 –1.0	8	1.8
9	M	47	Carcinoma, lung	0.1 –0.9	11	6.8

REFERENCES

1. Gal, E. M.; Fung, F. H.; and Greenberg, D. M. Studies on the Biological Action of Malononitriles. I. The Effect of Substituted Malononitriles on the Growth of Transplanted Tumors in Mice. Cancer Research, **12**:565–72, 1952.

Studies on the Biological Action of Malononitriles

II. Distribution of Rhodanese (Transulfurase) in the Tissues of Normal and Tumor-bearing Animals and the Effect of Malononitriles Thereon*

EMERY M. GAL,† FUNG-HAAN FUNG, AND DAVID M. GREENBERG

(Department of Biochemistry, School of Medicine, University of California, Berkeley, Calif.)

INTRODUCTION

The enzyme rhodanese, which catalyzes the formation of thiocyanate from cyanide and thiosulfate, is of interest to the cancer problem for reasons to be mentioned below. The administration of nitriles causes an increase in the thiocyanate level of the blood and in its urinary excretion. In seeking an explanation for the growth-retarding effect of certain substituted malononitriles and the lack of effect of others (4), it appeared logical to us to study the relations of this enzyme to tumors and the effect of the malononitriles thereon.

Mendel, Rudney, and Bowman (7) reported that rat tissues (including malignant tumors) which exhibit a high aerobic glycolysis contain negligible rhodanese activity, while tissues in which glycolysis is suppressed by oxygen have high rhodanese activity. The low rhodanese activity of tumor tissues has been reaffirmed by Rosenthal (8, 10), and low values were also found in many tissues with a normal aerobic metabolism. The above reports appeared only as abstracts without details or numerical values. Himwich and Saunders (5), in an investigation of the tissues of normal dogs, found disagreement between their results and those of the above-mentioned authors. On this point it should be mentioned that Rosenthal has noted that there are quite pronounced species differences in the activity of rhodanese of homologous tissues (8).

Mendel *et al.* (7), moreover, have suggested that, in tissues exhibiting aerobic glycolysis, the lack of rhodanese would permit an accumulation of cyanide which could account for the total or partial disappearance of the Pasteur effect. This hypothesis is of great potential interest for the field of cancer chemotherapy, as it suggests the possibility of altering the metabolic pattern of neoplastic tissue by appropriate inhibitors of the enzymes of glycolysis and respiration, an approach which has to some extent been exploited (1). The above hypothesis suggested that, through the administration of sufficient cyanide or nitriles, cellular respiration could be poisoned to a degree that would bring the metabolism of the tumor cell to a halt. It should be mentioned that a variety of alternate explanations have been offered to account for the lack or the inhibition of the Pasteur effect (3).

Nevertheless, it appeared worth while to explore the relation of rhodanese to the biological activity of the nitriles and, more generally, the significance of this enzyme for the cancer problem.

The questions raised by the discussion given above are: (*a*) Is the rhodanese activity of tumors really so low as to be totally insignificant? (*b*) Does continuous administration of malononitriles exert a noticeable influence on the rhodanese activity of the tissues of normal and tumor-bearing animals?

To obtain information on the several points raised, the distribution of rhodanese activity in the tissues of normal and tumor-bearing animals and the influence of malononitrile administration on the enzyme activity have been investigated. In addition, an endeavor has been made to explore the relation of this enzyme to the neoplastic process. The results obtained show that, while the rhodanese content of tumors, in general, is small, it is by no means inconsequential in comparison to other metabolically active tissues.

* This work was supported in part by grants from the National Cancer Institute, United States Public Health Service.

† U.S. Public Health Special Fellow, 1948–50.

Received for publication February 12, 1952.

MATERIALS AND METHODS

The tissues of normal and tumor-bearing strains, A, C3H, and C57 mice, and Long-Evans, Slonaker, and Sprague-Dawley rats were used. The tumors used were mouse carcinomas LCS, C3H-S, E 0771, mouse sarcomas S37, 6C3H-ED, and myeloid leukemia (Bar Harbor C1498), rat carcinoma 256 (Walker), and hepatoma produced by azo dye feeding. The mice were kept under identical conditions to those described in the previous paper (4). Both sexes were employed, as no significant difference in the rhodanese content of their tissue was detectable. In support of the observation by Rosenthal *et al.* (11), we found that the weight and age of the animals were more critical, since changes in the diet of the animals considerably influenced the enzyme content. All rats were kept on a Purina Laboratory Chow diet and used within a narrow range of weight.

The animals were killed by a blow on the head, the tissues were rapidly excised, put on ice, homogenized with 10 times their weight of distilled water, and filtered through cheese cloth. The homogenates were kept at 5° C. and were worked up within 12 hours of the preparation. We found that additions of the components in the following order gave good results, namely: 0.3 ml. of filtered tissue homogenate, 7.9 ml. phosphate buffer (pH 7.4), 0.5 ml. 0.1 M sodium thiosulfate, and 0.5 ml. 0.1 M potassium cyanide. The mixture was shaken for different time intervals (15 min.–2 hr.) at 37.5° C. The enzymatic activity was stopped at the desired time interval by adding 10 ml. of ferric nitrate solution, prepared according to the specification of Lavietes and his associates (6). The mixture was then diluted to 25 ml. with distilled water, mixed, centrifuged, and allowed to stand 15 minutes in darkness. The thiocyanate readings were made in a Beckman spectrophotometer at 520 mμ, and, from the optical density, the concentration was read off from the chart of a standard curve prepared by adding known amounts of a solution of potassium thiocyanate (anhydrous) to the tissue preparation in the absence of cyanide. Due to the fact that some reversibility of the reaction has been reported (8), the standard curve was also checked against a boiled homogenate, with and without cyanide. No significant difference was found between the standard curves established by the two different methods. The above-mentioned proportions and concentrations were used in all experiments unless otherwise stated. The rhodanese activity of the tissues is given in terms of mg SCN^-/gm or mg CN^- converted/gm of the tissue measured.

Whenever the number of animals in the experiments was reasonably large, the standard deviation (σ) of the determinations was calculated and reported in the tables.

RESULTS

The first experiments were performed to determine the effect *in vitro* of a number of nitriles on rhodanese activity. The results are shown in Table 1. In carrying out these experiments, amounts of the nitriles equivalent to the cyanide in experiment 2 were added and then incubated for the time intervals shown in the table. Thiosulfate and cyanide were then added, the enzyme mixtures were incubated for an additional 15 minutes, and the amount of thiocyanate formed was determined. The results show that none of the nitriles tested replaced cyanide as a substrate for the rhodanese in the homogenate enzyme system. Furthermore, these nitriles had no inhibitory effect on the enzyme, when compared to the incubations with either the enzyme and buffer, or the enzyme, buffer, and thiosulfate. In fact, it appears plausible that the nitriles might to some extent protect the enzyme against oxidation by tying up some of the trace metals. Furthermore, cyanide caused no inhibition of rhodanese in the homogenates, contrary to previous reports (5).

TABLE 1

THE EFFECT OF NITRILES ON RHODANESE*

No.	Inhibitor	Quantity† inhibitor (mg.)	mg SCN^- formed/gm tissue Time: 15′	30′	60′	120′
1	‡		0.490	0.270	0.179	0.129
2	KCN	3.25	0.790	0.805	0.465	0.410
3	CH_3CN	2.05	0.660	0.446	0.258	0.213
4	$CH_2(CN)_2$	1.65	0.677	0.587	0.307	0.311
5	C_2H_5OH: $C(CN)_2$	3.05	0.666	0.510	0.283	0.320
6	$Na_2S_2O_3$§		0.686	0.570	0.300	0.260
7	$Na_2S_2O_3$+KCN		1.150	1.230	1.235	1.235

* Assays were performed in systems composed of 0.3 ml. of homogenate in 7.9 ml. phosphate buffer (pH 7.4) + nitrile shaken for different times at 37.5° C., followed by 0.5 ml. 0.1 M $Na_2S_2O_3$ and 0.5 ml. 0.1 M potassium cyanide for another 15 minutes' interval. No. 7 is the complete system without nitrile used as a control.

† The amounts of CN group per dose of inhibitor administered were all equivalent to 50 μM of CN^-.

‡ Homogenate + buffer only.

§ S_2O_3 = 5.60 mg.

The other possibility, that the nitriles may have competitively displaced the cyanide, was ruled out in a series of experiments which showed that even twice the cyanide equivalent of the nitriles had no inhibitory effect on rhodanese. All the attempts to produce thiocyanate release from the nitriles *in vitro* either in homogenates or in tissue slices failed. These results made it necessary to turn to the intact animal to study the catabolism of the nitriles and the possible influence of the catabolic products on rhodanese.

The similarities between neoplastic and embryonic tissues led us to perform experiments to establish the time relations of the development of rhodanese activity in the growing embryo and, simultaneously, the effect of pregnancy on the con-

tent of this enzyme in the liver and kidney of the rat, particularly as no data were found in the literature. The results are shown in Table 2. It is interesting that the enzyme values in liver and kidney are both below the nonpregnant control averages of about 28 and 22 mg CN^- converted/gm of tissue, respectively, although it might be expected that pregnancy would provoke an increased response to detoxification mechanisms. The rhodanese activity of the whole embryo is of the same order of magnitude as found for tumor tissue, and lyzed individually. Two of the 3-month animals showed cirrhotic foci but no distinct areas of hepatoma even after 3 months of feeding. The rhodanese activity of the tissues of these animals did not differ from that of the controls, but the enzyme content of the cirrhotic parts of the liver was lowered slightly.

The animals which developed hepatomas showed decreased rhodanese levels. The lower values of the azo dye-fed animals during the early stages might be due to the initial lowered food intake. Later on,

TABLE 2

CHANGE OF RHODANESE ACTIVITY DURING FETAL DEVELOPMENT*

AGE OF EMBRYO (weeks)	WHOLE EMBRYO	EMBRYO LIVER	LIVER PREGNANT RAT	KIDNEY PREGNANT RAT
	(Av. mg CN^- converted/gm tissue)			
2	0.26±0.075	1.90±0.12	19.00±2.13	14.50±0.57
3	0.99±0.17	5.55±0.095	24.30±3.74	22.50±5.85
at birth	1.16±0.13	14.32±2.70	24.03±2.73	18.50±1.64

* Each group represents tissues and progeny of five female rats of an average weight of 300 gm. Enzyme determinations of the embryos were on the whole embryos including the placenta.

TABLE 3

EFFECT OF AZO-DYE TREATMENT ON THE RHODANESE ACTIVITY OF SPRAGUE-DAWLEY RATS*

PERIOD DYE ADMINISTRATION (weeks)	AV. WT. ANIMALS† (gm.)	BRAIN	TESTES	ADRENALS‡	KIDNEY	LIVER	LIVER TUMOR AREAS
		(Av. mg CN^- converted/gm tissue)					
2	90 (4)	0.47±0.10	0.76±0.29	0.96	6.18±1.90	8.44±1.40	
Control	100 (4)	1.26±0.45	1.71±0.83	1.70	15.60±2.16	20.73±2.51	
8	200 (5)	0.63±0.42	1.36±0.08	3.63	14.02±4.19	19.39±2.92	
Control	180 (5)	0.50±0.16	1.46±0.14	3.02	16.80±6.95	22.03±3.66	
12	233 (2)	1.19	1.59	2.47	22.70	28.80	17.90§±1.37
	260 (4)	0.52±0.12	1.11±0.51	3.05	14.92±1.89	18.15±2.08	2.97#±0.36
Control	270 (4)	0.60±0.09	1.72±0.33	2.90	17.00±2.34	28.20±3.76	

* The azo dye-treated animals were obtained through the courtesy of Dr. A. Clark Griffin, Dept. of Chemistry, Stanford University, Stanford, Calif.

† Figures in parentheses represent number of animals analyzed statistically.

‡ The adrenals of the animals were pooled.

§ Cirrhosis.

Well developed hepatoma.

the content in embryo liver is noticeably low, although it rises rapidly at birth. Determinations carried out without the placenta gave an increase in value of about 10 per cent.

The significance of rhodanese for the neoplastic process was studied by determining the changes in rhodanese activity of the tissues of Sprague-Dawley rats at different stages of advancing malignancy induced by azo dye feeding (3′-methyl-4-dimethylaminoazobenzene) and also by estimation of the rhodanese content of the tissues of normal and tumor-bearing mice.

The results, up to 3 months of azo dye feeding, are shown in Table 3. The livers of the azo dye-fed animals were carefully examined, and the unaffected and diseased portions were separated and analyzed when the initial shock of the treatment subsided, the difference in food intake became almost negligible.

In very advanced hepatoma tissues the rhodanese activity became significantly lowered. It is possible that the values for hepatoma are misleadingly low, as the necrotic parts were not removed prior to analysis. No rhodanese activity ever was found in any of the necrotic tissue of any of the rat or mice tumors investigated.

The above results, which made it evident that the azo dye treatment resulted in somewhat lowered tissue content of rhodanese, raised the question as to how transplanted tumors would influence the tissue levels of this enzyme. To this end the tissues of normal and Walker carcinoma 256-

bearing Slonaker rats were examined on animals in which the weights and the diet were carefully equalized. The Walker carcinoma was preferred, because of not only its good response to chemotherapeutic agents, but also its rapid growth and abundant vascularization. The latter made it the tumor-bearing mice. Table 5 shows the tabulated values of the rhodanese activity of the different strains of normal and tumor-bearing mice. In contrast to the observations with rats, the transplanted tumors of the mice did not appreciably influence the rhodanese content of the tissues analyzed. The efficiency of the cyanide conversion of the tumors was not much lower than that of either the brain or the spleen, and, in the case of the tumor LCS-A, it was even higher than the values for the brain and spleen of the control animals.

TABLE 4

THE RHODANESE ACTIVITY OF NORMAL SLONAKER AND WALKER CARCINOMA-BEARING RATS*

	CONTROL	TUMOR-BEARING
	(Av. mg CN^- converted/gm tissue)	
Tumor†		0.43±0.14
Brain	2.65±1.67	0.97±0.28
Kidney	22.20±3.76	14.20±3.16
Liver	28.35±3.80	19.40±4.23
Adrenals	1.58‡	1.65‡
Testes	4.38±2.54	2.20±1.64
No. of determinations	12	9

* The rats were all male, 5 months of age, and weighed from 200 to 250 gm.

† The necrotic parts included.

‡ Adrenals were pooled.

The above results bring us back to our first question and the specific problem of whether the rhodanese content of the tumors is really insignificant. In certain types it is decreased, impaired perhaps, but it is not insignificant. If one considers that most of these tumors grow to 3–5 gm., then their power to convert cyanide into thiocyanate becomes considerable. The specific activity per gram of tumor weight was, no doubt, even higher at the early period of the growth when the necrotic parts of the tumor did not as yet cause much dilution. This is not merely a question of semantics, because it means that the tumors can handle all the cyanide that either gets there or forms there. If this were not true the brain, spleen, adrenals, and the other tissues with low rhodanese content would all be seriously flooded with cyanide before it could accumulate in the tumors. Furthermore, it is not correct to compare the tumor values to those of the liver and the kidneys. Histologically, these experimental transplanted tumors are derived either from mammary tissue, lymph glands, or from the spindle cells of the connective tissue. As we know, these tissues show low rhodanese content themselves. The observations presented herein do not seem to subscribe to the proposed theory of Mendel *et al.* (7). The same reasoning applies to embry-

TABLE 5

RHODANESE ACTIVITY IN SOME OF THE TISSUES OF NORMAL AND TUMOR-BEARING MICE

GROUP	AGE OF ANIMALS (months)	AV. WT. ANIMALS (gm.)	AGE OF TUMOR (days)	BRAIN	KIDNEY	LIVER	SPLEEN	TUMOR*	NO. DETERMINATIONS
				(Av. mg CN^- converted/gm tissue)					
C3H-S	3	23.0	17	0.66±0.10	2.01±0.16	11.11±0.65	0.85±0.20	0.52±0.17	7
Control†	3	23.0		0.70±0.40	5.48±4.95	13.76±0.34	1.12±0.19		5
6C3H-ED	9	32.7	17	0.57±0.21	3.78±1.22	11.60±4.34	0.99±0.14	0.70±0.22	5
Control	9	32.0		0.47±0.10	3.20±0.11	11.15±0.12	0.49±0.09		5
C57 E 0771	9	36	17	0.82±0.11	3.27±1.67	11.20±1.88	0.70±0.19	0.68±0.20	7
(Myeloid leukemia) C57	9	30.5	8	0.62±0.05	3.83±0.66	11.10±0.62	0.72±0.03	0.63±0.19	5
Control	9	27.0		0.71±0.03	4.49±0.15	12.60±2.59	0.96±0.19		5
A-S37	8	25.0	17	1.03±0.42	5.05±3.12	9.95±3.92	0.62±0.32	0.72±0.27	9
A-LCS	8–10	27.2	15	0.70±0.03	3.51±2.82	7.94±5.19	0.98±0.01	1.32±0.79	9
Control	8–10	25.5		0.81±0.17	6.89±1.70	13.36±1.24	0.66±0.22		5

* The necrotic parts included.

† Control below each tumor-bearing group denotes the same strain and age of mice.

tumor of choice for the study of the supply of cyanide and the draining-off of thiocyanate in tumors.

Table 4 contains the results obtained with tumors after 2–3 weeks of growth. The trend toward lower rhodanese values in the tissues of the Walker 256 carcinoma-bearing rats is unmistakable. Nevertheless, the results must be accepted with caution until it is established more conclusively that the effect observed was actually a function of the malignancy.

Rats, because of their size, were convenient animals for the enzyme and detoxication studies. However, since mouse tumors were used in the experiments investigating the growth-retarding effect of malononitriles, it was important to estimate the rhodanese content of the tissues of normal and

onic tissue. Even though the liver of a 2-week-old embryo is so small that manipulation under a binocular is necessary, on the basis of its rhodanese activity it could transform close to 2 μg of cyanide/mg/15 minutes. This is not insignificant when it is considered that an average adult rat excretes between 60 and 160 μg. of thiocyanate daily, which, at a most conservative estimate, corresponds to not less and perhaps more than 10 per cent of the total thiocyanate content of the rat body.

In order to answer our second question regarding the release of cyanide from the malononitriles as being responsible for the growth-retarding effect, the rhodanese content of animals treated with malononitriles was investigated.

The results of these observations are tabulated in Table 6. This table indicates that neither malononitrile nor *p*-nitrobenzalmalononitrile had any significant effect on the rhodanese content of the tissues of the C3H-S-bearing mice. The values of the control animals in this series agree well with those of the C3H animals given in Table 5.

TABLE 6

THE EFFECT OF INJECTION OF MALONONITRILE ON THE RHODANESE CONTENT OF C3H-S-BEARING MICE*

	Av. wt. animals (gm.)	Tumor	Liver (Av. mg CN^- converted/gm tissue)	Kidney	Adrenals†
Malononitrile	25.0	0.79±0.08	10.82±1.05	3.13±0.92	1.50
p-Nitrobenzalmalononitrile	23.5	0.63±0.04	11.05±0.86	2.50±0.79	1.48
Control‡	25.0	0.88±0.13	9.91±1.18	3.59±0.85	1.65

* Duration of treatment was 16 days. Administered daily dose was 40 μg. of malononitrile and 150 μg. of *p*-nitrobenzalmalononitrile in 0.05 ml. asym. propylene glycol. These quantities are less than the previously employed therapeutic doses given in Table 3, Paper I (4). The reason for lowering the dosage was to decrease the mortality. However, it still represents a total quantity 20 times the lethal dose of sodium cyanide. Each group consisted of fifteen animals.

† Adrenals were pooled.

‡ The controls were C3H-S-bearing untreated animals.

DISCUSSION

The intriguing hypothesis of Mendel and his associates (7) attributes the partial or total abolition of the Pasteur effect in some tissues with high aerobic glycolysis to the accumulation of cyanide. They reason that this accumulation of cyanide is the result of the practical absence of rhodanese activity in those tissues. In brain, spleen, and ileal mucosa, where rhodanese occurs in sufficient quantities, they argue that glycolysis is suppressed in oxygen almost to "the maximum anticipated by the Meyerhof quotient," whereas in tumors the aerobic glycolysis would not be affected because of the presence of enough cyanide to inhibit most of the respiration. These arguments gain some support through the observations of Warburg (12) that cyanide favors aerobic glycolysis of cells and that embryonic tissues can become malignant through the anoxia caused by cyanide administration to tissue cultures. A serious objection against attributing the lack of the Pasteur effect in tumors to an insufficiency of the rhodanese content arises from the observation made by Rosenthal (9, 10) and the work reported here.

If cyanide is to inhibit cellular function to a significant degree, without causing death of the animal, then the total amount of cyanide produced cannot exceed 26–70 μg/kg/15 min. Of course, it is known that *in vivo* death would be due to the rapid poisoning of the cytochrome system of the brain. This again does not signify that *in vitro* the rhodanese would not be able to detoxify that lethal amount of cyanide.

We know that there is a small but steady rate of thiocyanate formation in the body. Also, it may well be that the continuous presence of very minute amounts of cyanide (for example in vitamin B_{12}) may have some very important cellular metabolic function. The likelihood would still exist, however, because of the different cyanide potentialities of the various tissues, for cyanide to accumulate at a greater rate than it could be removed by the circulation, particularly in the case of the tumors. However, our results strongly indicate that an accumulation of effective amounts of cyanide in any of the tissues will be counteracted because of the presence of rhodanese, even if the enzyme occurs only in small quantities. It has also been shown that not even a continuous steady supply of cyanide would appreciably affect any of the tissues *in situ* of the animals investigated. It is also evident, at least from the values obtained in the tumors the authors have investigated, that one can hardly correlate the rhodanese activity with the Meyerhof quotient, as tissues, like the tumors with a high Meyerhof quotient, had just about as much rhodanese activity as the tissues with a low Meyerhof quotient. In view of the experimental data pre-

sented in this paper, and also from other unpublished evidence, the authors feel that, as far as the tumors analyzed by them were concerned, the interesting hypothesis of Mendel and his associates cannot be supported. In this respect the conclusions reached agree with certain of Rosenthal's observations (9, 10).

The reasons for an anomalous Pasteur effect in tumors must, therefore, be due to other causes.

SUMMARY

1. Malononitriles did not inhibit the rhodanese activity in tissue homogenates, nor did they competitively displace the cyanide.

2. The rhodanese content of fetal tissue increased in amount with development and at birth. The rhodanese activity of the tissue of the mother was not significantly influenced by pregnancy.

3. Animals fed 3′-methyl-4-dimethylaminoazobenzene showed a slight decrease in the rhodanese activity coincident with the development of hepatoma. The hepatoma itself showed a significantly damaged rhodanese activity, while the surrounding unimpaired liver tissues continued functioning normally.

4. The Walker rat carcinoma 256 produced a general lowering of the rhodanese activity in the tissues, which could not be correlated with either loss of weight or a lowered food intake. This was not the case in the tissues of mice with transplanted tumors.

5. A detailed analysis of different mouse transplanted tumors and the tissues of both the normal and tumor-bearing mice of strains A, C3H, and C57 revealed that the tumors do not have less rhodanese content than their homologous tissues or of some of the vital organs. Liver and kidney, in comparison, possess a disproportionately high activity of rhodanese that well fits in with their function of being the prime sites of cyanide detoxification.

6. Administration of malononitrile and *p*-nitrobenzalmalononitrile to C3H-S-bearing mice for a period of 16 days did not influence the rhodanese activity of the tissues or that of the tumors.

REFERENCES

1. BLACK, M. M.; KLEINER, I. S.; and BOLKER, H. Glycolytic Enzyme Inhibitor Therapy in Human Malignant Neoplasia. Cancer Research, **9**:314–19, 1949.
2. BURK, D. A Colloquial Consideration of the Pasteur and Neo-Pasteur Effects. Cold Spring Harbor Symp. Quant. Biol. **7**:420–59, 1939.
3. DICKENS, F. Anaerobic Glycolysis, Respiration, and the Pasteur Effect. In SUMNER, J. B., and MYRBÄCK, K. (eds.), The Enzymes, **2** (part 1): 624–83, 1951.
4. GAL, E. M.; FUNG, F. H.; and GREENBERG, D. M. Studies on the Biological Action of Malononitriles. I. The Effect of Substituted Malononitriles in the Growth of Transplanted Tumors in Mice. Cancer Research, **12**:565–72, 1952.
5. HIMWICH, W. A., and SAUNDERS, J. P. Enzymatic Conversion of Cyanide to Thiocyanate. Am. J. Physiol., **153**:348–54, 1948.
6. LAVIETES, P. H.; BOURDILLON, T.; and KLINGHOFFER, K. A. The Volume of the Extracellular Fluids of the Body. J. Clin. Investigation, **15**:261–68, 1936.
7. MENDEL, B.; RUDNEY, H.; and BOWMAN, M. C. Rhodanese and the Pasteur Effect. Cancer Research, **6**:495, 1946.
8. ROSENTHAL, O. The Distribution of Rhodanese. Fed. Proc., **7**:181, 1948.
9. ———. Are a "Tumor-Like" Metabolism and Rhodanese Deficiency Biochemical Characteristics of Mucosa of the Small Intestine? Acta Union internationale contre cancer, **6**:307, 1948.
10. ———. Rhodanese Activity and Tumor Metabolism. Cancer Research, **9**:546, 1949.
11. ROSENTHAL, O.; ROGERS, C. S.; VARS, H. M.; and FERGUSEN, C. C. Arginase, Adenosinepyrophosphatase, and Rhodanese Levels in the Liver of Rats. J. Biol. Chem., **185**:669–80, 1950.
12. WARBURG, O. The Metabolism of Tumours, pp. 141–42. London: Constable & Co., Ltd., 1930.

Mitochondrial Population of Mammalian Cells

II. Variation in the Mitochondrial Population of the Average Rat Liver Cell during Regeneration. Use of the Mitochondrion as a Unit of Measurement*

CLAUDE ALLARD, GASTON DE LAMIRANDE, AND ANTONIO CANTERO

(Montreal Cancer Institute, Research Laboratories, Notre-Dame Hospital, Montreal, Canada)

INTRODUCTION

The mitochondrial population of the average normal adult rat liver cell has been estimated in this laboratory and found to be approximately 2,500 (2). Preliminary results have shown that the number of these large granules in a rat liver cell may fluctuate in different physiological conditions (2). The average liver tumor cell contains about 1,400[1] and the precancerous cell 1,700 mitochondria.[2] In regenerating liver the decrease per average cell appeared so evident (2) that the phenomenon was thought to be a characteristic change occurring in stimulated growth process. The present investigation seems to favor this working hypothesis. In the normal growth process, no gross variation in the number of mitochondria per average cell is detectable by our method of estimating mitochondria.

METHODS

A total of 80 albino adult Wistar rats[3] of both sexes, weighing 120–220 gm., was partially hepatectomized (66 per cent) according to the method of Higgins and Anderson (3). Before and after the operation the animals were fed Purina Fox Chow and water ad libitum. At periods of 1, 2, 3, 4, 6, 8, 12, and 20 days after partial removal of the liver, three animals or more were killed, the livers excised, weighed, homogenized in 0.25 M sucrose, and fractionated as previously described (2) to isolate three cellular particulate fractions. Mitochondria were counted in the separate nuclear (N), mitochondrial (M) and supernatant (S) fractions under the phase microscope with a Petroff-Hausser bacteria counter (2). It has been shown that the summation of the number of mitochondria found in the (N), (M), and (S) fractions equals the number found in the original homogenate of normal or regenerating liver. The number of nuclei per gram of fresh liver was determined by the technic employed previously by Price and Laird (5). The micro-Kjeldahl procedure was employed for total nitrogen determinations

* This investigation is being supported by a grant from the National Cancer Institute of Canada.

[1] Submitted for publication, Canad. J. Med. Sc.

[2] Submitted for publication, Canad. J. Med. Sc.

[3] Purchased from a local dealer.

Received for publication February 16, 1952.

RESULTS

The total number of mitochondria, which was estimated by adding the number of these granules found in the (N), (M), and (S) fractions, may be determined directly in the homogenate but with greater visual difficulty (2). For this reason, and also because the number of mitochondria in the separate fractions was needed in concurrent studies, they were counted in the isolated fractions (2).

Table 1 shows the mean number of nuclei and mitochondria per gram of fresh adult regenerating and immature rat liver and the respective mean number of mitochondria per average cell. For control purposes, the values previously published for normal rat livers (2), the mean values obtained from liver lobes removed during partial hepatectomies, and the values obtained from two livers of sham-operated animals, one day post-laparotomy, are given. The total number of animals used is indicated in parentheses.

Marked fluctuations in the number of cells per gram of fresh liver during regeneration, observed by other investigators (5), were not noted in this laboratory. However, unperfused livers were used for the cell counts, and a different strain of rats was adopted in this laboratory; furthermore, the diet differed from the one used by these authors (5). Thus, only a slight increase above the normal level was observed during the fourth day after partial hepatectomy. The weight of the livers at this period of regeneration was usually double that of the first day post-hepatectomy. The total number of cells per whole regenerating liver, calculated

from the number of nuclei per gram of liver (Table 1) and the weight of the respective liver, is found to be about 40 per cent of the normal level during the first day and 80 per cent during the fourth day. These approximate results agree with Abercrombie's findings (1). The process of rapid growth which occurred during the 4 days following hepatectomy does not necessarily seem to be accompanied by variations in the number of cells per gram of fresh liver.

The mean total number of mitochondria per gram of fresh tissue (Table 1) appeared to decrease abruptly during the first day after partial hepatectomy and to remain thereafter at this twelfth day the average liver cell had regained its normal number of mitochondria. Assays performed 2 months after partial hepatectomy revealed that the liver cell was normal with respect to the number of mitochondria. In the liver of immature rats the number of mitochondria per cell was practically normal—the decrease noted on a per gram fresh tissue basis seemed to be due to a low cell population of the respective livers.

The percentage of mitochondria present in the (N) fraction and the total nitrogen content of the mitochondrial fraction on a per gram fresh tissue and on a per mitochondrion basis, during regeneration, are illustrated in Table 2. It is striking that

TABLE 1

MEAN NUMBER OF MITOCHONDRIA PER AVERAGE CELL OF REGENERATING AND IMMATURE RAT LIVER

Material	No. nuclei per gram fresh tissue $\times 10^{-6}$	No. mitochondria per gram fresh tissue $\times 10^{-10}$	No. mitochondria per cell*
Controls:			
Normal liver (adult rats)	129 (42)	33† (11)	2,550
Liver removed at hepatectomy‡	130 (15)	31.4 (15)	2,420
Sham-operated. One day post-laparotomy	115 (2)	36.5 (2)	3,170
Regenerating liver:			
Days post-hepatectomy:			
1	135 (15)	24.2 (10)	1,800
2	130 (6)	25.9 (12)	1,990
3	134 (3)	23.0 (9)	1,720
4	142 (8)	25.0 (8)	1,760
6		27.0 (3)	
8	134 (4)	25.3 (8)	1,890
12	111 (4)	26.7 (7)	2,380
20	120 (3)	25.8 (3)	2,150
Immature livers of rats:			
(6–8 weeks)	107 (7)	25.5 (7)	2,380

* Assuming mononuclear cells.

† Previously published.

‡ Pool of fifteen partial livers removed at hepatectomy.

lower level throughout the period studied. Immature liver contained about the same number of mitochondria per gram as regenerating adult liver.

Apparently, during the first day after laparotomy there was an increase in the number of mitochondria. The liver lobes removed at hepatectomy contained about the same number of mitochondria per gram of fresh tissue as previously observed (2). In fact, significant variations in the number of cells and mitochondria in different groups of Wistar rats have not yet been observed.

The probable variation in the mitochondrial population during liver regeneration is illustrated in Table 1. During the first day after partial removal of the liver, the number of mitochondria per cell decreased abruptly and remained at a low level until the eighth day. Thereafter, the number of mitochondria seemed to increase slowly. By the

the number of mitochondria in the (N) fraction was increased when the particulates were isolated from regenerating liver. This increment was not entirely due to a decrease in the total number of mitochondria but also to an absolute increase in the number of mitochondria found in the (N) fraction. Eight determinations on normal liver (2) gave a mean value of 3.5×10^{10} mitochondria for an equivalent of 1 gm. of fresh tissue. A value of about 4.7×10^{10} was obtained for liver in the process of regeneration, which corresponded to approximately a 30 per cent increase. On a per gram fresh tissue basis, it was found that the TNC of the mitochondrial fraction differed significantly from the control only during the third, fourth, and sixth day post-operative. On a per mitochondrion basis, a significant difference was observed only on the first day of regeneration.

DISCUSSION

Surprisingly, the marked decrease in the number of mitochondria per average cell of regenerating liver remained constant between the first and the eighth day after partial hepatectomy. It seemed that no direct relationship existed between cell division and the mitochondrial population. On the contrary, it appeared that the mitochondria might be formed slowly in the daughter cells—i.e., subsequent to cell division. However, it is possible that, in rapidly growing tissue, the rate of mitochondrial formation might be insufficient, as compared to the rate of cell division. When liver growth is normal, as in immature rats, the mitochondrial population seems normal. However, a smaller number may exist in the newly divided cells; but it is impossible by our method to detect such a phenomenon, because newly divided cells are scarce in such livers.

The significant increase in the nitrogen content in the average mitochondrion during the first day of regeneration, when correlated with other changes occurring in the cell during interphase, such as the DNA accumulation in the nucleus (5), suggests a variation in the chemical constitution of the mitochondrion during the process of cell division. Furthermore, the fact that the absolute number of mitochondria present in the isolated (N) fraction of regenerating liver is increased suggests a possible physical change—e.g., a variation in the size or density of some or all of the mitochondria present in the regenerating liver cells.

Statistically, after the second day of regeneration the variations in the TNC of the (M) fraction on a per gram tissue-equivalent basis seem to reflect the respective number of mitochondria. In fact, after the second day of regeneration no significant fluctuations exist on a per mitochondrion basis. It should be noted that when the TNC is expressed on a per gram fresh tissue basis, instead of a per mitochondrion basis, a different relationship exists. As already suggested (2), the mitochondrion as a unit seems more appropriate in expressing content or concentration of a chemical constituent of the mitochondrial fraction.

The investigation of the mitochondrial population of the average liver cell during chemical carcinogenesis in rat liver by 4-dimethylaminoazobenzene revealed that this particular azo dye might be responsible for a decrease in the number of these particles during the very early phase of carcinogenesis. This decrease of mitochondria in the average cell is similar to that occurring during stimulated growth of the liver after partial hepatectomy.

TABLE 2

MEAN CORRECTED* TOTAL NITROGEN CONTENT (TNC) OF THE MITOCHONDRIAL FRACTION AND OF THE AVERAGE MITOCHONDRION DURING RAT LIVER REGENERATION

Material	No. animals	Percentage of mitochondria present in the (N) fraction	Mg. (TNC) in (M) fraction per equivalent of 1 gm. fresh tissue	Mg. (TNC) $\times 10^{-12}$ per mitochonrion
Normal liver	11	10.6	7.4	22.3
Liver removed at hepatectomy†	15	13.4	7.0	22.4
Regenerating liver:				
Days post-hepatectomy:				
1	7	18.9	6.8	28.0‡
2	12	18.9	6.5	25.1
3	11	18.3	4.3‡	18.7
4	15	18.8	5.5‡	22.2
6	4	14.4	6.0‡	22.1
8	10	20.5	6.7	26.7
12	7	18.9	6.6	24.8
20	7	20.5	6.5	25.2

* Corrected for the percentage of mitochondria present in the (N) fraction (2).
† Pool of fifteen partial livers removed at hepatectomy.
‡ The difference from control value is significant, with a probability exceeding 0.95.

SUMMARY

1. The mitochondrial population of the average rat liver cell during regeneration was investigated by counting the mitochondria isolated from liver homogenates prepared in 0.25 M sucrose.

2. The population seemed to decrease in the average cell when the liver was in the process of stimulated growth after partial hepatectomy.

3. Possible chemical and physical changes in the average mitochondrion during regeneration of the liver have been discussed.

4. The mitochondrion as a unit for measurement of chemical constituents present in the mito-

chondrial fraction has been proposed as a more appropriate basis than other methods of expression.

ACKNOWLEDGMENTS

We wish to extend our gratitude to Miss Denise Plante, for her technical assistance, and to Mr. Yvon Grandchamp, statistician.

REFERENCES

1. ABERCROMBIE, M., and HARKNESS, R. D. The Growth of Cell Populations and the Properties in Tissue Culture of Regenerating Liver of the Rat. Proc. Roy. Soc., London, s.B., **138**:544–61, 1951.
2. ALLARD, C.; MATHIEU, R.; DE LAMIRANDE, G.; and CANTERO, A. Mitochondrial Population in Mammalian Cells. I. Description of a Counting Technic and Preliminary Results on Rat Liver in Different Physiological and Pathological Conditions. Cancer Research, **12**:407–12, 1952.
3. HIGGINS, G. N., and ANDERSON, R. M. Experimental Pathology of the Liver; Restoration of the Liver of the White Rat Following Partial Surgical Removal. Arch. Path. **12**: 186–202, 1931.
4. LANDING, B. H.; SEED, J. C.; and BANFIELD, W. G. The Effects of a Nitrogen Mustard, Tris(2-chloroethyl)amine, on Regenerating Rat Liver. Cancer, **2**:1067–74, 1949.
5. PRICE, J. M., and LAIRD, A. K. A Comparison of the Intracellular Composition of Regenerating Liver and Induced Liver Tumors. Cancer Research, **10**:650–58, 1950.

The Effect of Inherited Factors, the Milk Agent, and Physiological State on Phosphorus Metabolism of Endocrine Glands of Mice*†

S. Albert and Ralph M. Johnson

With the Technical Assistance of Miss Ruth Hoste

(*Richard Cohn Radiobiology Laboratory, Detroit Institute of Cancer Research, Detroit 1, Mich.*)

It has been amply demonstrated that at least three factors—inherited susceptibility, a hormonal influence, and the milk agent—are concerned with the development of spontaneous mammary carcinomas in mice (3). Information on the mechanisms of action of these factors is, however, inadequate. Aub, Karnofsky, and Towne (2) found no difference in the urinary excretion of estrogens and 17-ketosteroids between low-cancer mice (C57) and high-cancer mice (C3H). On the other hand, Samuels and Bittner (5) demonstrated a decrease in the amount of free ketosteroids in the feces of mice bearing the milk agent, although the excretion of estrogens remained unaltered from the levels observed in the same strain without the agent.

Recent preliminary observations in this laboratory indicate that inherited factors, the milk agent, and physiological state play an important role in the phosphorus metabolism of the endocrine glands of inbred mice and suggest avenues for further study. The strains studied include the DBA, DBA−,[1] and C57 black (subsequently referred to as C57BL).

MATERIALS AND METHODS

Virgin and breeder female mice of the low-cancer DBA− and C57BL strains, and of the high-cancer DBA strain, and pregnant DBA mice were employed. All mice were 4–8 months old. The virgin and breeder mice weighed 18–27 gm., and the pregnant mice weighed 28–37 gm. Breeding mice were used 10 days after delivery of their second litters, which had not been permitted to suckle, and pregnant mice were taken 3–5 days prior to the expected termination of their third pregnancy. All mice were fed Purina Mouse Chow and water ad libitum.

A single injection of radioactive phosphorus,[2] as $Na_2HP^{32}O_4$ containing 29 μc., was administered subcutaneously to each mouse. The animals were sacrificed in groups of four by cervical dislocation 1, 4, 17, or 48 hours after administration of the P^{32}. Ovaries, adrenals, thyroids, and pituitaries were removed, freed of nonglandular tissue, washed with distilled water to remove excess blood, blotted, and weighed immediately on a Roller-Smith torsion balance. Each organ was then digested in 1–2 cc. of 2 N NaOH, warmed gently on a sand bath. An aliquot of each digested organ was taken for the determination of total phosphorus, and a second aliquot from the same material was used for the assay of radioactivity according to methods previously described (1). Radioactivity was expressed as a concentration coefficient, calculated in the following manner:

$$\frac{\text{counts/min in organ}/\mu\text{g phosphorus in organ}}{\text{counts/min injected}/\mu\text{g of body weight}} \times 10^{-1}$$

The phase of the estrous cycle at the time of each animal's death was determined by microscopic examination of a portion of the vagina, which had been fixed in Bouin's solution, sectioned, and stained with hematoxylin and eosin.

RESULTS

Organ Weights

A comparison of the organ weights of DBA and DBA− mice revealed several differences (Table 1). The ovaries of virgin DBA mice were heavier than those of DBA− virgins, while in the breeders, DBA− mouse ovaries weighed more than those of the DBA mice. The ovaries of both DBA and DBA− virgin and breeder mice and the thyroids of DBA and DBA− virgin mice were heavier than those of the corresponding C57BL mice. On the other hand, the adrenals and pituitaries of virgin

* Supported by grants from the National Cancer Institute of the National Institutes of Health, Public Health Service; the American Cancer Society; the S. S. Kresge Foundation; and the Michigan Cancer Foundation.

† A preliminary report of this work was presented before the American Association for Cancer Research, April 11–13, 1952, and was published in abstract form in the Scientific Proceedings of that meeting.

[1] The DBA− mice differ from the DBA in that they are free of the milk agent. They were Caesarean-born and foster-nursed by Bd (4) mothers, and carried subsequently by brother-sister mating as Dr. William S. Murray's stock 2.

Received for publication February 23, 1952.

[2] The radioactive phosphorus used in these experiments was furnished by the Oak Ridge National Laboratory, Oak Ridge, Tenn.

and breeder C57BL mice weighed more than did these glands in the corresponding DBA and DBA− mice except the pituitaries of the DBA− breeders.

Breeding did not affect the weight of adrenals, thyroids, and pituitaries in the mice of the three strains examined, but increased the weight of the ovaries in DBA− and C57BL mice, and decreased their weight in DBA mice. Pregnancy was accompanied by an increase in the weight of all organs over that found in the breeders. This observation is in keeping with the findings of other workers (6).

The Phosphorus Content of Endocrine Organs

When the phosphorus contents of the organs of the DBA and DBA− mice were compared, no major differences were found except in the thyroids of virgin mice (Table 2). The thyroids of the virgin DBA animals contained less phosphorus than the corresponding glands of the DBA− virgins.

The ovaries of both virgin and breeder DBA and DBA− mice contained less phosphorus than those of the C57BL mice. On the other hand, greater amounts of phosphorus appeared in the adrenals of mice of the first two strains than in the corresponding glands of the C57BL animals. Less phosphorus was found in the thyroids of DBA and DBA− virgin mice than in these glands of the C57BL counterparts. These differences, however, were not found in the breeders. No clear-cut differences were observed in the phosphorus content of the pituitary gland.

No major differences were observed in the phosphorus content of the endocrine glands of virgin mice when compared to that in the same organs of breeders. The sole exception occurred in the thyroids of DBA mice. In the latter strain, breeding was accompanied by an increase in the phosphorus content of the thyroids.

A diminished phosphorus content was found in the organs of the pregnant DBA mice when compared to that of the organs of DBA breeders. This decrease was greatest in the thyroid and pituitary glands.

A comparison of the relative phosphorus content of the organs of mice of each of the three strains (Table 2) revealed additional differences. This is best seen by arranging the organs in the following order of decreasing phosphorus content:

DBA and DBA− virgins	DBA and DBA− breeders	C57BL virgins	C57BL breeders
adrenal	adrenal	ovary	pituitary, ovary
ovary	ovary	adrenal	adrenal
pituitary	pituitary	pituitary	thyroid
thyroid	thyroid	thyroid	

P^{32} Uptake by Endocrine Organs

The ovaries, adrenals, and thyroids appeared to accumulate P^{32} more rapidly than the pituitaries (Charts 1–4). The maximum uptake occurred in most organs within 4 hours after administration of

TABLE 1

ENDOCRINE ORGAN WEIGHTS*

Strain	Physiological state	No. animals	Ovaries	Adrenals	Thyroids	Pituitaries
DBA	virgin	20	17.6±1.2	4.4±0.3	2.6±0.2	1.4±0.1
	breeder	17	12.7±0.5	3.8±0.2	2.3±0.2	1.3±0.1
	pregnant	17	15.2±0.6	5.3±0.3	3.0±0.2	1.7±0.1
DBA−	virgin	16	12.8±0.8	4.3±0.2	2.3±0.2	1.0±0.1
	breeder	16	17.4±0.8	4.2±0.3	2.4±0.3	1.4±0.2
C57BL	virgin	16	5.7±0.2	5.9±0.4	1.8±0.1	1.7±0.1
	breeder	16	7.7±0.3	5.4±0.3	2.0±0.2	1.6±0.2

* Figures represent average weights of moist tissues in milligrams and the standard errors.

TABLE 2

TOTAL PHOSPHORUS CONTENT OF ENDOCRINE ORGANS*

Micrograms phosphorus/100 mg tissue

Strain	Physiological state	No. animals	Ovaries	Adrenals	Thyroids	Pituitaries
DBA	virgin	20	346± 9.2	367±21.4	156±21.9	289±37.0
	breeder	17	342± 7.2	389±18.9	236±21.9	328±32.8
	pregnant	17	329± 6.5	357±13.4	158±10.7	248±22.3
DBA−	virgin	16	341±11.5	383±13.2	209±18.5	312±17.9
	breeder	16	330± 8.3	396±23.7	223±27.5	296±48.0
C57BL	virgin	16	370±11.6	335±13.1	296±31.4	285±19.8
	breeder	16	375±13.5	316±15.9	248±17.7	352±43.1

* Figures represent average values with standard errors.

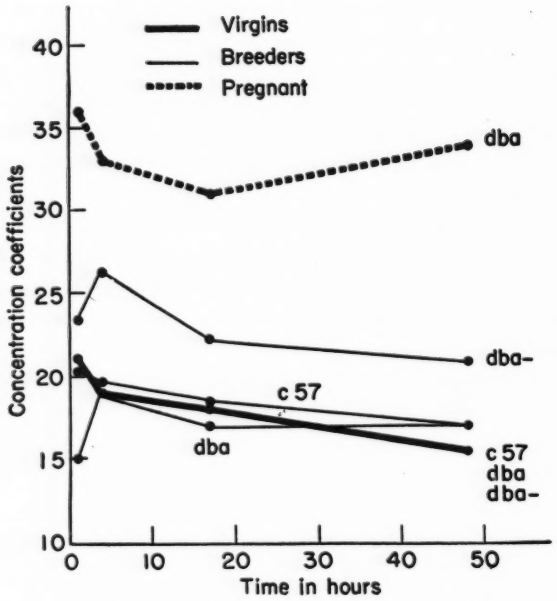


CHART 1.—Uptake of P^{32} in mouse ovaries. Each point represents the average of four observations, the average deviation being less than 16 per cent.

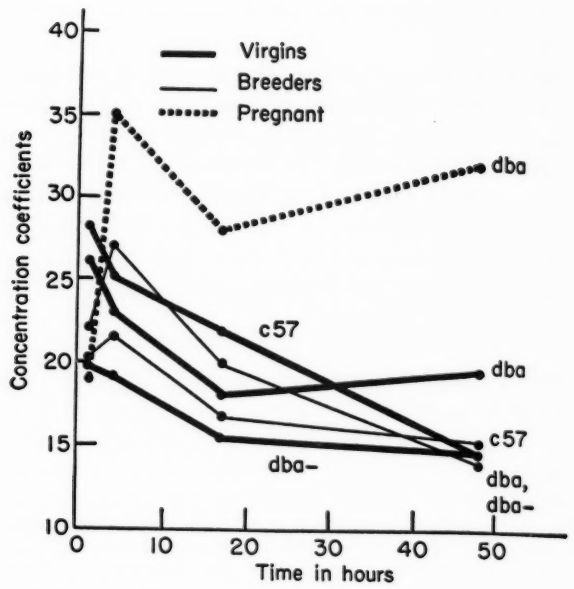


CHART 3.—Uptake of P^{32} in mouse thyroids. Each point represents the average of four observations, the average deviation being 23 per cent.

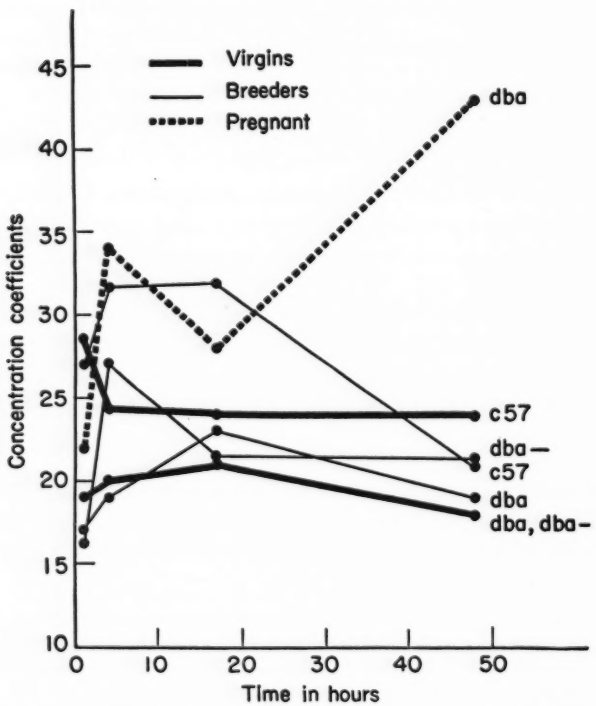


CHART 2.—Uptake of P^{32} in mouse adrenals. Each point represents the average of four observations, the average deviation being 23 per cent.

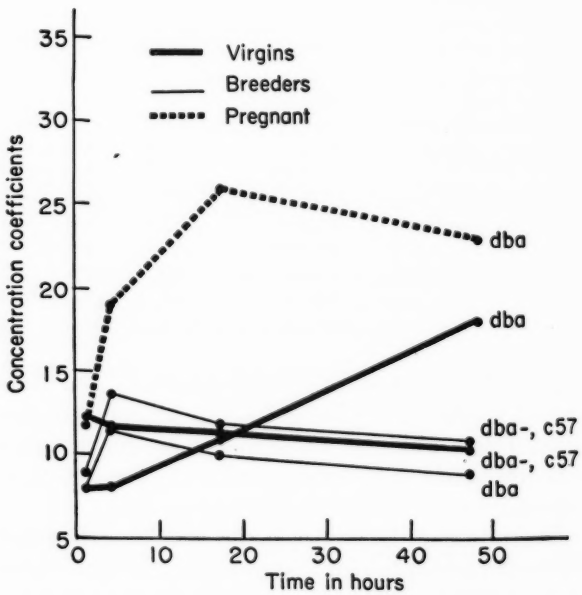


CHART 4.—Uptake of P^{32} in mouse pituitaries. Each point represents the average of four observations, the average deviation being less than 20 per cent.

the radioactive isotope. However, maximum uptake occurred much later in the adrenals of the DBA mice, and in the pituitaries of the virgin and pregnant DBA mice.

No differences were observed in the incorporation of P^{32} into the ovaries of the virgin mice of the three strains. Breeding was accompanied by an increased P^{32} uptake in the ovaries of the DBA− mice, but not in these organs of DBA and C57BL mice. Among the breeders, P^{32} uptake was most rapid in the ovaries of the DBA− mice and slowest in those of the DBA mice (Chart 1).

The accumulation of P^{32} in the adrenals of virgin DBA mice was similar to that in the adrenals of DBA− virgin mice, and in both it was slower than that in the C57BL virgin mouse adrenals. Breeding did not alter the incorporation of P^{32} into the adrenals of DBA mice but increased it in these organs in both DBA− and C57BL animals. The most rapid uptake occurred in the adrenals of the C57 strain mice, and the slowest occurred in those of the DBA mice (Chart 2).

The accumulation of P^{32} in the thyroids of the DBA virgin mice was similar to that of the C57BL virgin mice and was more rapid than in the thyroids of the DBA− mice. Breeding increased the uptake of P^{32} in the DBA− mouse thyroids, and decreased it in the thyroids of the C57BL mice. Thus, in breeders the uptake of P^{32} in DBA and DBA− mouse thyroids was similar and higher than that in C57BL mouse thyroids (Chart 3).

The incorporation of P^{32} into the pituitaries of virgin DBA− and C57BL mice was strikingly similar and initially higher than that in the pituitaries of the DBA virgins. However, the latter continued to accumulate P^{32} throughout the experiment and possessed higher concentration coefficients after 17 hours. No clear-cut effect of breeding on P^{32} uptake by pituitaries was observed (Chart 4).

DISCUSSION AND CONCLUSIONS

Differences were observed when the weights of the organs of DBA and DBA− mice were compared to those of the C57BL mice. Further differences were found when the amounts of phosphorus in the ovaries, adrenals, and thyroids of virgin DBA and DBA− mice, and in the ovaries and adrenals of breeder DBA and DBA− mice, were compared to those in the corresponding organs of C57BL mice. Inasmuch as the DBA and DBA− mice are believed to be genetically identical, and are genetically different from the C57BL mice, these differences must be ascribed to inherited factors.

Inherited factors also influenced the accumulation of P^{32} in the ovaries, adrenals, and thyroids, inasmuch as differences were observed when the uptake of P^{32} in these glands of DBA and DBA− mice were compared to that in the corresponding glands of the C57BL mice. These differences were, in certain instances, modified by the presence of the milk agent. Such a modification was observed in two cases. The uptake of P^{32} in the thyroids of DBA− virgins was less than that of this gland in C57BL virgins, and the uptake of P^{32} in the ovaries of DBA− breeders was higher than in C57BL breeder mouse ovaries. In contrast, these differences were not observed in these organs of the corresponding DBA mice in the presence of the milk agent.

A comparison of the concentrations of phosphorus and of the data on radioactivity of the organs of DBA and DBA− mice demonstrated that the presence of the milk agent was accompanied by profound changes in phosphorus metabolism of the endocrine glands. In the presence of the milk agent there was a decrease in the phosphorus content of the thyroids of virgin animals, an increased P^{32} uptake in the thyroids of virgin animals, a decrease in the P^{32} uptake in the ovaries and adrenals of breeder mice, and, initially, a decrease in P^{32} uptake in virgin animal pituitaries.

The milk agent was also associated with an increase in the weight of virgin mouse ovaries and pituitaries and with a decrease in the weight of the ovaries in breeders.

The data suggest that the change from the virgin to the breeder state influenced phosphorus metabolism. This was manifested by an increase in P^{32} uptake by all the organs of DBA− mice, by the adrenals of C57BL mice, and by a decreased P^{32} uptake in the thyroids of C57BL mice. However, the most striking changes in phosphorus metabolism attributable to a change in physiological state were those which accompanied pregnancy. This condition was characterized by a relatively large increase in the P^{32} uptake in all glands examined (Charts 1–4). Such a change could be due either to an increase in organ size or to increased endocrine activity. An increase in the weight of all organs of the pregnant animals over that found in the breeders was observed. However, this increase in weight was accompanied by a decrease in phosphorus concentration. This would indicate either that pregnancy was accompanied by a loss of phosphorus from these organs or that the increase in weight was due to a nonphosphorus-containing material such as water. If our present understanding is correct, that endocrine glands do not elaborate phosphorus-containing compounds, the latter explanation would seem more logical.

The increased P^{32} uptake accompanying pregnancy would then be interpreted as due to increased endocrine activity.

These experiments indicate that the physiological state of the animal is an important condition for the expression of differences due to inherited factors and the milk agent. The change from the virgin to the breeder may either accentuate the differences or cause them to disappear, depending on the organ concerned. For example, in the thyroids the differences in phosphorus content that were due to inherited factors were only apparent in the virgin state. On the other hand, the influence of inherited factors on the uptake of P^{32} by the ovaries was only apparent in the breeder state.

To determine which phosphorus fractions are concerned in the above changes, experiments are in progress involving fractionation of endocrine organs into various phosphorus-containing fractions.

SUMMARY

1. Radioactive phosphorus, as $Na_2HP^{32}O_4$, was administered to virgin and breeder mice of the DBA, DBA−, and C57BL strains, and to pregnant mice of the DBA strain. The mice were sacrificed 1, 4, 17, or 48 hours after the administration of the P^{32}, and the total phosphorus and P^{32} content of the ovaries, adrenals, thyroids, and pituitary glands were determined.

2. Inherited factors influenced the phosphorus content of ovaries, adrenals, and thyroids, the P^{32} uptake in the ovaries, adrenals, and thyroids, and the weights of all endocrine organs studied.

3. The presence of the milk agent was associated with an increase in the weight of the ovaries of the virgins, a decrease in the weight of these glands in breeders, and a decrease in P^{32} uptake in the ovaries and adrenals of breeder animals. It was also associated with a decrease in the amount of phosphorus in the virgin animal thyroids, and with an increased P^{32} uptake in virgin animal thyroids. Lastly, it was associated with an increased weight of and an initially decreased P^{32} uptake in virgin mouse pituitaries.

4. The change from the virgin to the breeder state was accompanied by a change in ovarian weight and by an altered P^{32} uptake in certain organs of DBA, DBA−, and C57BL mice. Pregnancy was accompanied by a moderate increase in endocrine organ weight and by a large increase in P^{32} uptake in all endocrine glands examined.

REFERENCES

1. Albert, S.; Johnson, R. M.; and Cohan, M. S. Phosphorus Metabolism in Resting and Pregnancy-stimulated Mammary Glands and Spontaneous Mammary Gland Carcinomas of Mice. Cancer Research, **11**:772–76, 1951.
2. Aub, J. C.; Karnofsky, D.; and Towne, L. E. Sex Hormone Excretion Rates in High and Low Tumor Strains of Mice. Cancer Research, **1**:737, 1941.
3. Moulton, F. R. (Ed.). A Symposium on Mammary Tumors in Mice. Am. Assoc. Adv. Sc., No. **22**, 1945.
4. Murray, W. S., and Little, C. C. Extra-chromosomal Influence in Relation to the Incidence of Mammary and Non-mammary Tumors in Mice. Am. J. Cancer, **27**:516–18, 1936.
5. Samuels, L. T., and Bittner, J. J. Excretion of Steroids and the Feces of Mice of Various Strains with and without the Mammary Tumor Milk Agent. Am. Assoc. Cancer Research, Inc., 38th Annual Meeting, 1947.
6. Selye, H. Textbook of Endocrinology. Montreal, Canada: Acta Endocrinologica, Université de Montréal Press, 1947.

The Nature of Mammalian Lymphosarcoma Transmission by Isolated Chromatin Fractions

GEORGE KLEIN

(Wallenberg Laboratory for Experimental Cytology, Institute for Cell Research, Karolinska Institutet, Stockholm, Sweden)

In a recent publication, Stasney, Paschkis, and Cantarow (6) reported the successful transmission of the Murphy rat lymphosarcoma by inoculation of chromatin fractions isolated from the neoplasm according to the procedure of Claude and Potter (1). Although they could not exclude contamination of these fractions with intact living cells, they considered such a contamination to be highly improbable and supposed that the tumors appearing after subcutaneous and intramedullary injections of the chromatin fractions represented a neoplastic transformation of lymphocytes in the recipient animals, induced by the inoculated fraction or by some of its components.

Whether tumors developing after the inoculation of tumor preparations that are presumably free of viable cells represent a proliferation arising *de novo* in the injected host or are due to contamination of the material with viable tumor cells can be decided by the use of tumor material that is homologous to an animal strain of known genetic composition, according to the procedure of Law (4).

It seemed of interest to repeat the experiments of Stasney *et al.* with tumors homologous to highly inbred mouse strains. The procedure of Law was then employed to detect whether the tumors produced by the injection of isolated chromatin fractions are of the same genetic constitution as the original tumor or whether they have the genetic characteristics of the host in which they developed.

MATERIALS AND METHODS

Two strains of transplantable mouse lymphoma were used, one homologous to the DBA strain, described and studied by Goldie and Felix (2), and the 6C3H-ED lymphoma of the C3H strain (5). Both tumors were kept in the strains of origin, where the percentage of takes was nearly 100 per cent. The DBA lymphoma failed to take in 50 C3H and 20 C57 black mice, or regressed completely after a short initial growth period. The 6C3H-ED lymphoma failed to take or regressed in 30 Strong A and twenty C57 black mice. Both lymphomas were carried by intraperitoneal passages of ascitic fluid. When marked abdominal swelling was present, the animals were sacrificed, and their ascitic fluids and solid intra-abdominal tumors were collected. The solid tissue was minced and mixed with the ascitic fluid, until a dense pulp was obtained. From this point on, the procedure of Stasney *et al.* (6) for breaking the cells with sand and, subsequently, the method of Claude and Potter (1) for preparing the chromatin fraction were meticulously followed; the latter is the same as that employed by Stasney *et al.* The chromatin fraction was suspended in saline and injected subcutaneously and intraperitoneally as follows: (*a*) the DBA lymphoma into DBA × C3H F_1 hybrids and DBA × C57 black F_1 hybrids; (*b*) the 6C3H-ED lymphoma into A × C3H F_1 hybrids and into C3H × C57 black F_1 hybrids (Tables 1 and 2). From an aliquot of each chromatin fraction fresh smears were prepared, fixed with alcohol-ether (1:1) and stained by the Papanicolaou and the Feulgen methods. The animals were observed for tumor or ascites development, and the appearing tumors were re-injected subcutaneously and intraperitoneally into similar F_1 hybrids and into both parent strains of the particular F_1 hybrid in which the tumor arose. Histologic examinations have been routinely made of all tumors, while ascitic fluids were examined by Papanicolaou smears.

Received for publication March 25, 1952.

RESULTS

The results obtained are shown in Table 1 (DBA lymphoma) and Table 2 (6C3H-ED lymphoma).

Although the chromatin fractions were examined on stained smears as mentioned above, no evidence was found microscopically for the presence of whole cells or whole nuclei in the fields examined. The microscopic appearance of the fractions was very similar to that of Murphy lymphosarcoma chromatin fraction, as shown by the microphotograph of Stasney *et al.* (6).

Inoculation of the chromatin fractions of either lymphoma led to the development of solid tumors in a number of animals, as seen in Tables 1 and 2. Ascites was not produced after intraperitoneal inoculation, with the exception of one case. The tumors from the DBA lymphoma appeared 13–27 days after inoculation, and in the case of the 6C3H-ED lymphoma after 10–23 days. They grew progressively and led to the death of the host in all cases. Survival times ranged from 31 to 58 days and from 23 to 55 days, respectively, for the two tumors. No differences could be detected between

the morphological structure of the original tumors and that of those tumors developing after injection of the chromatin fraction.

Re-inoculation of the solid tumors that developed in the susceptible F_1 hybrids into both susceptible and resistant parent strains and into similar F_1 hybrids gave unequivocal and consistent results for both tumors. All inoculations led to tumor development in members of the susceptible parent strain (strain of origin of the tumor) and in similar F_1 hybrids, while no progressive growth was observed in members of the resistant parent strain.

DISCUSSION

After transplantation into genetically controlled material, the behavior of the tumors that arose in suitable F_1 hybrids upon inoculation of the chromatin fractions is proof that these tumors had the same genetic constitution as the original tumor from which the fractions were isolated and not the genetic constitution of the F_1 hybrid host. (For a detailed discussion see Law [4]). Tumors arising in F_1 hybrids are known not to be transplantable to any of the parent strains but only to similar F_1 hybrids. These findings make improbable the tentative conclusion of Stasney *et al.*, who suggested that such chromatin fractions possibly induce a neoplastic transformation in the injected host, as the cells of the developing tumor should in such a case be expected to have the same genetic constitution as the F_1 hybrid in which the tumor arose. The most probable explanation of the findings is a contamination of the chromatin fraction with surviving cells from the original tumor.

The failure, with one exception, of the chromatin fractions to produce ascites tumors after intraperitoneal inoculation into the same animals in which solid tumors developed after subcutaneous injection is in good agreement with the previously established fact (3) that low cell-doses of ascites-producing tumors do not lead to typical ascites tumors but only to solid tumor development.

TABLE 1

INJECTION OF DBA LYMPHOMA CHROMATIN FRACTION

Genotype of Animals Injected with Chromatin Fraction	Total no.	Subcut. tumor	Ascites	After Re-inoculation of Developing Tumors Progressive Growth in (tumor/total) DBA	F_1 Hybrids	C57 bl.	C3H
DBA×C3H F_1 hybrids	10	7	1	10/10	10/10		0/10
DBA×C57 bl. F_1 hybrids	10	5	0	10/10	10/10	0/10	

TABLE 2

INJECTION OF 6C3H-ED LYMPHOMA CHROMATIN FRACTION

Genotype of Animals Injected with Chromatin Fraction	Total no.	Subcut. tumor	Ascites	After Re-inoculation of Developing Tumors Progressive Growth in (tumor/total) C3H	F_1 Hybrids	A	C57 bl.
A×C3H F_1 hybrids	10	5	0	10/10	10/10	0/10	
C57 bl.×C3H F_1 hybrids	10	6	0	10/10	10/10		0/10

SUMMARY

The experiment of Stasney *et al.* (6) on tumor production by the injection of chromatin fractions isolated from a rat lymphosarcoma was repeated, with two transplantable mouse lymphomas that originated and grew in genetically controlled animal strains. The development of lymphomas after subcutaneous inoculation of chromatin fractions has been confirmed, but evidence has been presented to show that the tumors which result do not possess the genetic constitution of the host in which they arise, but that of the original tumor. The most probable explanation of these findings is contamination of the chromatin fractions with surviving cells, although, as in the experiments of Stasney *et al.*, intact cells or nuclei could not be detected microscopically in any of the fractions studied.

REFERENCES

1. Claude, A., and Potter, J. S. Isolation of Chromatin Threads from the Resting Nucleus of Leukemic Cells. J. Exper. Med., 77:345–53, 1943.
2. Goldie, H., and Felix, M. D. Growth Characteristics of Free Tumor Cells Transferred Serially in the Peritoneal Fluid of the Mouse. Cancer Research, 11:73–80, 1951.
3. Klein, G. The Production of Ascites Tumors in Mice and Their Use in Studies on Some Biological and Chemical Characteristics of Neoplastic Cells. Uppsala: Almquist och Wiksells Boktryckeri AB, 1951.
4. Law, L. W. Effects of Low Temperatures on Mammary Carcinomas with and without the Mammary Tumor Milk Agent. Cancer Research, 11:795–800, 1951.
5. Lits, F. J.; Kirschbaum, M.; and Strong, L. C. Action of Colchicine on Transplanted Malignant Neoplasm in Mice of C3H Strain. Am. J. Cancer, 34:196–203, 1938.
6. Stasney, J.; Cantarow, A.; and Paschkis, K. E. Production of Neoplasms by Injection of Fractions of Mammalian Neoplasms. Cancer Research, 10:775–82, 1950.

Effects of the Application of Carcinogens to Exposed Gastric Mucosa in the Rat*

SIDNEY D. KOBERNICK, EDNA W. TOOVEY, AND D. R. WEBSTER

(Department of Pathology and the Experimental Surgical Laboratories, McGill University, Montreal, Canada)

INTRODUCTION

Gastric adenocarcinoma is a rare, spontaneous tumor in animals (1, 2, 7, 8, 21) and is difficult to produce experimentally with carcinogens (3, 4, 14, 15). Feeding experiments with carcinogens have all too frequently produced neoplastic lesions only in the forestomach (6, 18) or in regions other than the stomach (5, 11, 12). Van Prohaska, Brunschwig, and Wilson (20) have pointed out the difficulty of keeping a carcinogenic agent in the stomach for a sufficient length of time for it to act locally, or even to bring this agent into intimate contact with gastric mucosa normally covered with mucus. In order to overcome these difficulties, Stewart and associates (14–17), together with other investigators (3, 4), have introduced carcinogens into the submucosa of the stomach by injections and by threads impregnated with carcinogens. The yield of true gastric malignancies based on the criteria of Klein and Palmer (17) is low by these methods, and until the tumor is large enough to palpate through the anterior abdominal wall its existence remains undetected. It was hoped that by applying carcinogens locally to a pedicled portion of gastric mucosa, exteriorized and fixed to the anterior abdominal wall of the rat, these difficulties would be surmounted.

MATERIALS AND METHODS

Piebald male rats of the Royal Victoria Hospital colony were prepared with an exteriorized pedicled portion of gastric mucosa anchored to the anterior abdominal wall by an operating technic previously described (19). The portion of gastric mucosa usually measured 1.5 × 1 cm. Two rats were kept in each cage throughout the experiment and were fed Purina Fox Meal ad libitum from the time they were weaned. Water was also supplied ad libitum. These rats were divided into groups and treated in the following manner:

1. Thirty rats were kept as untreated controls.

A second group of untreated controls, comprising 50 animals, was killed, in pairs, at predetermined intervals to obtain fresh histological material for studying the development of the flaps.

* This work was supported by a grant-in-aid from the National Cancer Institute of Canada.

Received for publication April 23, 1952.

2. Thirty rats were treated with the vehicle, polyethylene glycol 300 (PEG 300), which was used to dissolve the carcinogens. This was painted on the exposed gastric mucosa 3 times a week with a No. 6 camel's hair brush after the mucosa had been wiped gently with gauze. PEG 300 was used for this work, because it was found to be a suitable solvent for carcinogenic hydrocarbons, nonirritating, stable, soluble in water, and nontoxic (9, 10, 13).

3. Thirty rats were painted 3 times a week with a 0.25 per cent solution of 20-methylcholanthrene (MCA) in PEG 300.

4. Thirty rats were painted 3 times a week with a 4 per cent solution of 2-acetylaminofluorene (2AAF) in PEG 300.

5. Thirty rats had threads, impregnated with 20-methylcholanthrene, inserted into the submucosa of the flaps on the tenth day after operation.

All these animals were treated until death, which, in some cases, occurred after more than a year.

Post mortem examinations were done on all rats found dead. Tissues from all the animals were fixed in 10 per cent formalin, Zenker's, and Susa's solutions, and were stained with eosin and hematoxylin. Additional sections of the gastric flaps were stained with Southgate's mucicarmine stain and periodic acid-Schiff stain.

RESULTS

The flap of gastric mucosa, fixed to the abdominal wall by sutures, first appeared to become hyperemic. Some of the flaps ulcerated and disappeared in a few days, but most regenerated and formed polypoid masses of pink mucosa on the anterior abdominal wall. The mucosa was constantly wet with mucus and manifested, at irregular but frequent intervals, areas of ulceration and crusting. The flaps reached considerable size in some animals that survived for long periods, some attaining 4.5 cm. in diameter from an original flap 1–1.5 cm.

When examined microscopically, at implantation, the flaps consisted of an intact portion of the stomach wall, placed on the muscle of the abdominal wall with the mucosal epithelium closely approximated to the skin epithelium (Figs. 1 and 2). Within 1 week, ulceration of the mucosa was seen, with acute purulent inflammation extending well into the lamina propria, obscuring the muscularis mucosae, with marked edema of the smooth muscle of the stomach wall (Figs. 3 and 4). At this stage, early proliferation of the squamous epi-

thelium adjacent to the graft appeared. By the second week, the ulceration was very extensive, and very little intact gastric mucosa was evident. That which remained appeared as atypical, low cuboidal, proliferating epithelium, with the more differentiated cells being almost entirely mucous in type (Fig. 5). The inflammation persisted in the third week but showed more subacute characteristics with infiltration of plasma cells and deposition of some collagen and proliferation of capillaries (Fig. 8). The proliferating gastric glands showed dilatation and formation of small cystic spaces, while the smooth muscle of the stomach wall was all but completely obscured by the deeper formation of inflammatory connective tissue (Fig. 7). The squamous epithelium at the margin of the gastric flap was definitely hyperplastic but showed no tendency to overgrow the flap or to penetrate beneath the gastric mucosa (Fig. 6).

The gastric epithelium continued to proliferate throughout the fourth, fifth, and sixth weeks, by which time the regeneration was usually complete and formed a polyploid mass overhanging the skin edges. Many of the glands showed well marked cystic dilatation (Fig. 9). The underlying submucosa was fibrotic and vascular, with a variable amount of plasma cell infiltration (Fig. 8). Throughout the course of the experiment the flaps were subject to repeated ulceration of the surface and inflammation of the underlying tissue, so that regeneration of portions of the epithelium appeared to be in constant progress. However, the destruction of the mucosa was never so extensive as in the earliest stages.

After the second month, the proliferating gastric epithelium, now entirely mucoid in character, formed deep penetrations into the submucosa which had the appearance of adenomas (Figs. 9 and 10); but serial sections demonstrated that these were invariably connected with the overlying surface epithelial glands. Furthermore, these formations were usually near the overhanging edges of the polyp-like growth of the flap. These deeply penetrating proliferations of the surface epithelium did not attain a depth below the original thickness of the implanted flap and have never yet been seen in the muscle of the abdominal wall. They tended to be somewhat more numerous with the progress of time. These formations could not be interpreted as true neoplasms, but rather as the result of continuous proliferation arising from the repeated ulcerating infections that occurred.

At 3 months, spicules of bone appeared in the deeper layers of some of the flaps (Fig. 11). These were interpreted as metaplastic bone formation secondary to chronic inflammation. After 10–12 months, the recurring infections tended to be superficial, and the absence of deep inflammation allowed the flap to become thinner and to approach the original thickness. However, the smooth muscle of the stomach wall disappeared completely (Fig. 12).

The changes described above were paralleled exactly by those occurring in the experimental groups treated with carcinogens (Fig. 12). No trace of the implanted MCA-impregnated threads was ever seen in the flaps, although remnants of the suture material used to fix the flaps to the anterior abdominal were often present, along with a foreign-body reaction to them. These were seen in both the carcinogen-treated and in the untreated control groups. *In no instance was any true adenoma or carcinoma seen.*

DISCUSSION

Transplanting flaps of gastric mucosa to the anterior abdominal wall of rats has demonstrated that the flaps can survive as gastric mucosa, although the parietal cells tend to disappear, and the mucosa is almost entirely mucous in type. The flap does not become overgrown by the skin. This provides a means of direct study of the mucosa and a controllable means of applying carcinogens or other substances. The gastric mucosa placed on the anterior abdominal wall is subject to repeated trauma from the floor of the cage, resulting in extensive and repeated ulceration of the surface epithelium, followed by marked inflammatory changes and an almost continuous regenerative activity on the part of the mucosa. In the process of regeneration, deep proliferations of mucosal glands which have the appearance of adenomas occur, but serial sections demonstrate that these are connected to the surface mucosa and cannot therefore be regarded as true neoplasms. Furthermore, these glands have never been seen beyond the confines of the original implant; this would appear to be the minimum criterion for considering them neoplastic growths.

SUMMARY AND CONCLUSIONS

1. Experiments in which flaps of gastric mucosa of 200 rats were transplanted to the muscle of the anterior abdominal wall were described. These animals were divided into: two groups of untreated controls; one group treated by the implantation of threads impregnated with 20-methylcholanthrene in the flaps; one group in which the flaps were painted with 20-methylcholanthrene; one in which the flaps were painted with 2-acetylaminofluorene; and one group painted with polyethylene glycol 300, the vehicle used to dissolve the carcinogens.

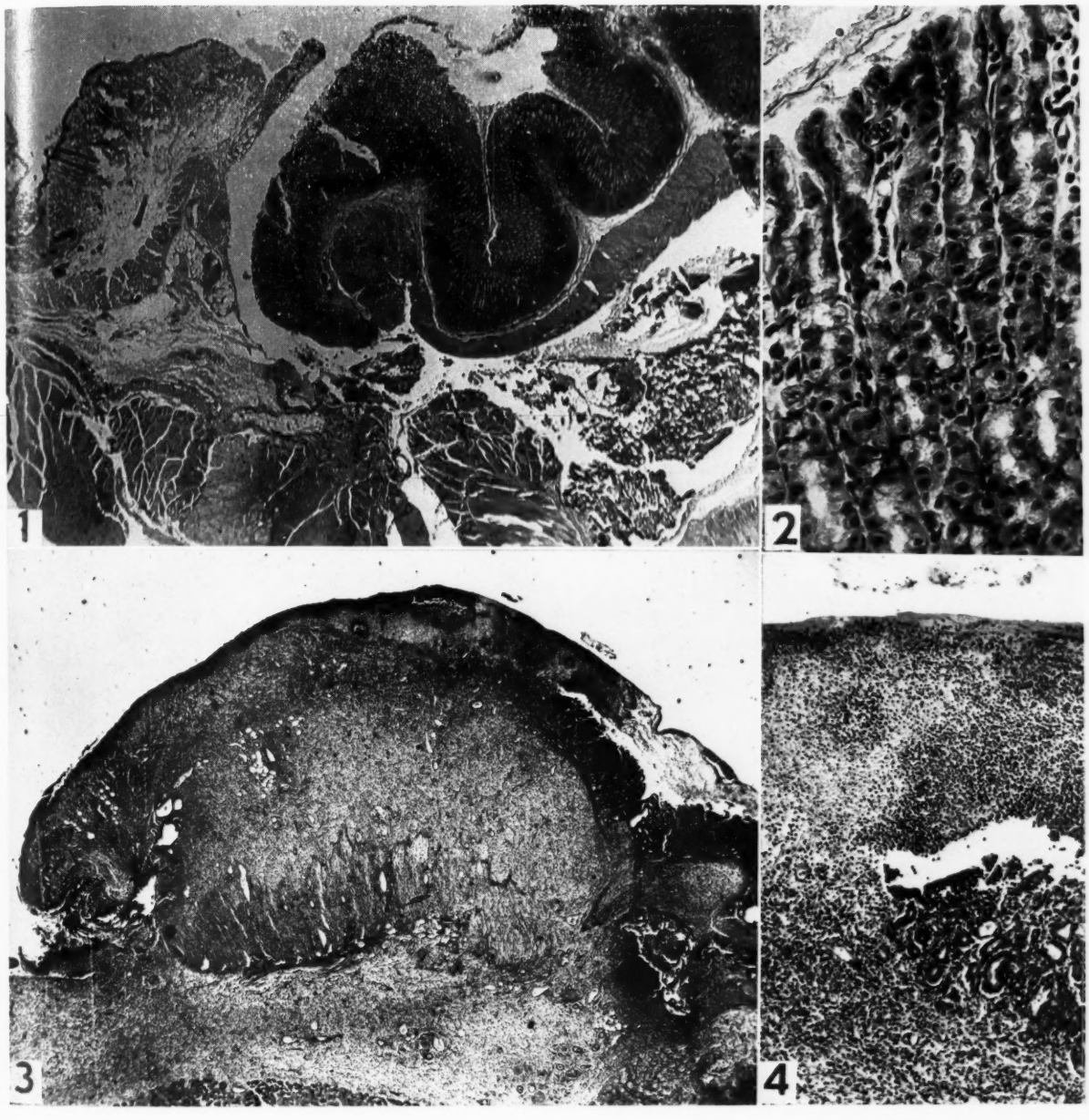

1
2
3
4

FIG. 1.—Gastric flap (Rat 299-R) immediately after implantation, showing skin at left with portion of whole stomach wall lying on the muscle of the anterior abdominal wall. Hematoxylin and eosin. ×20.

FIG. 2.—Detail of mucosa of flap shown in Figure 1. Note the mixture of parietal and mucus cells of normal gastric mucosa. Hematoxylin and eosin. ×225.

FIG. 3.—Gastric flap (Rat 433) 1 week after transplantation showing marked ulceration of the surface epithelium and almost complete replacement by acute inflammatory reaction. The submucosa is greatly thickened, and the muscle fibers of the wall of the stomach are separated by the exudate which also extends into the layer between the flap and the anterior abdominal wall. Hematoxylin and eosin. ×18.

FIG. 4.—Higher magnification of the border between intact epithelium and the exudate shown in Figure 3, showing the polymorphonuclear character of the exudate. Hematoxylin and eosin. ×79.

FIG. 5.—Gastric flap (Control rat 3-430) 2 weeks after transplantation, showing regenerating mucosa growing into the exudate and regenerated mucus type of epithelium. The exudate beneath the epithelium contains capillaries and lymphocytes and plasma cells in addition to polymorphonuclear leukocytes. Hematoxylin and eosin. ×137.

FIG. 6.—Squamous epithelium of skin adjacent to flap (Control rat 3-430) 2 weeks after transplantation showing marked hyperplasia. Infiltration of inflammatory exudate may be seen beneath it and around its junction with the flap at the extreme right. Hematoxylin and eosin. ×81.

FIG. 7.—Gastric flap (Control rat 3-440) 3 weeks after implantation, showing the cystic dilatation of the regenerating epithelium. Deep islands of gastric mucosa may be seen at the lower portions of the figure. Periodic acid-Schiff stain. ×30.

FIG. 8.—Detail of Figure 7, showing the mucus nature of the regenerated epithelium and the submucosa composed of newly formed fibrous tissue and capillaries infiltrated with lymphocytes and plasma cells. Periodic acid-Schiff stain. ×210.

5

6

7

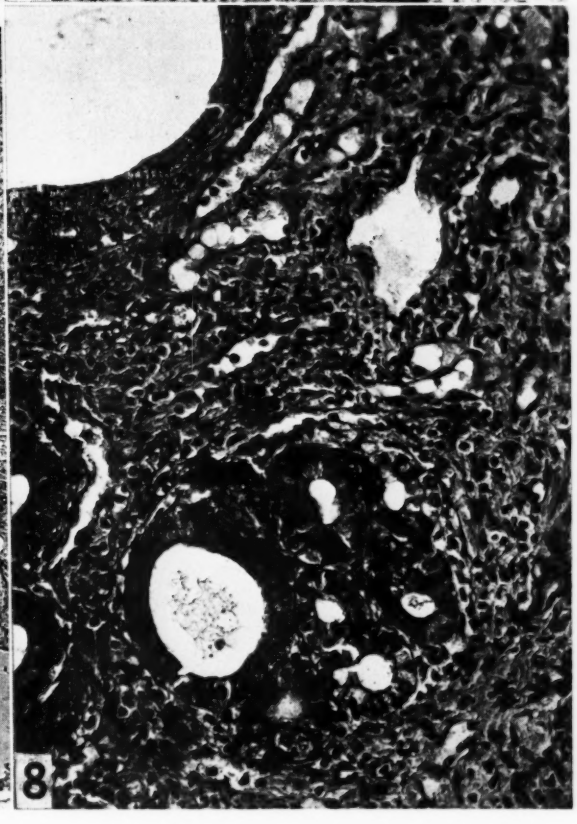
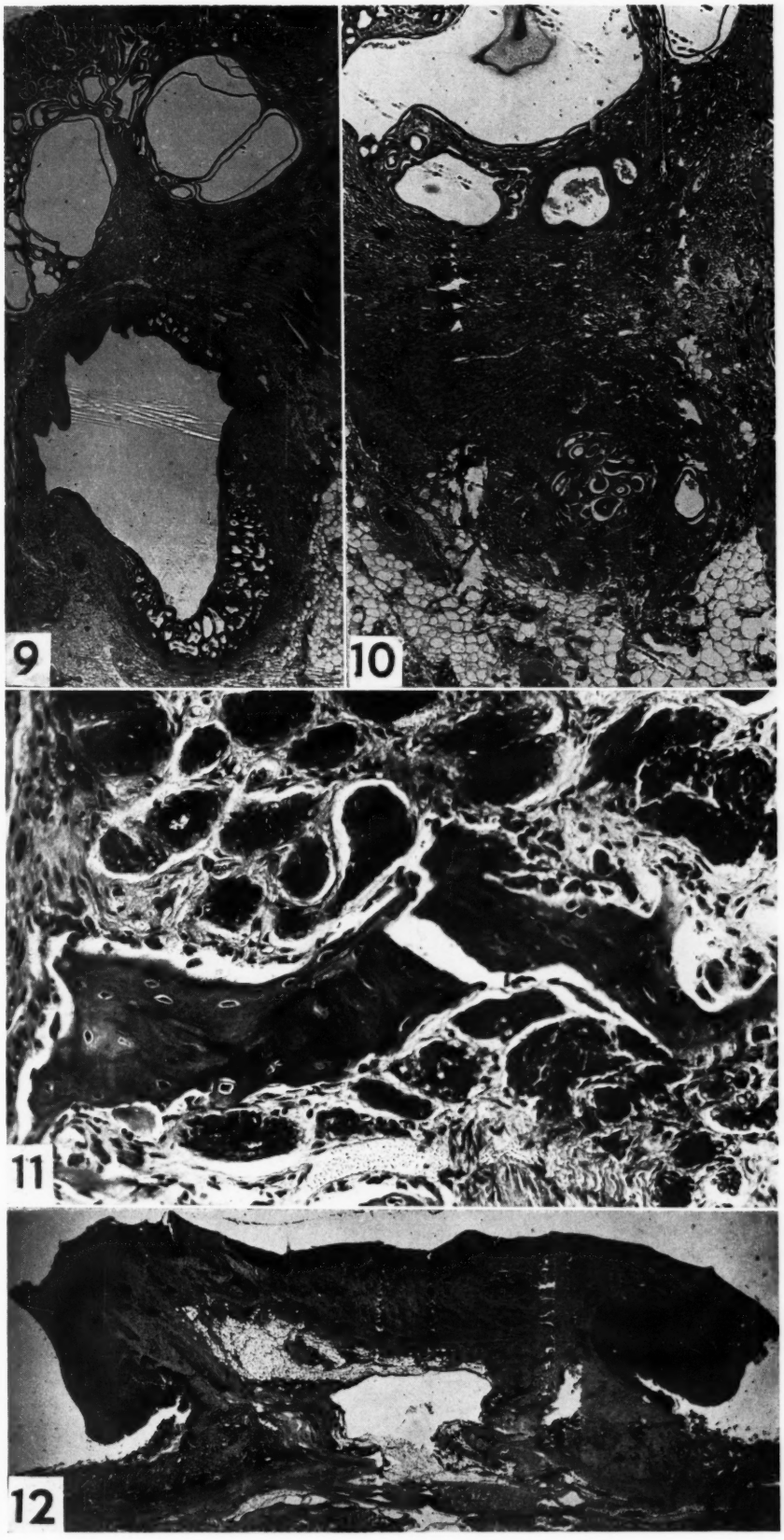

8

FIG. 9.—Portion of gastric mucosa (Rat 416, untreated control) 4 months after implantation showing deep almost isolated cystic adenoma-like formation. Neighboring sections demonstrated the connection with the mucosa clearly. Hematoxylin and eosin. ×20.

FIG. 10.—Isolated group of gastric acini (Rat 394, normal control) in deepest layers of flap 6 months after transplantation. Connection with mucosa demonstrated in neighboring sections. Hematoxylin and eosin. ×35.

FIG. 11.—Metaplastic bone formation in mucosa of normal control rat (421) 3 months after transplantation. Periodic acid-Schiff stain. ×200.

FIG. 12.—General configuration of flap 11½ months after transplantation (220). Although this rat was treated by painting with MCA, the extensive ulceration and crusting of the mucosa in the central portion is similar to that seen in normal controls as is the degree of general growth indicated. The absence of any overgrowth of skin epithelium is also evident. Mucicarmine. ×5.


9
10
11
12

The tissues were studied histologically as the animals died. Observations extended beyond 1 year in some animals.

2. The mode of development of the flaps was described in detail, with emphasis on the characteristic occurrence of marked, recurrent ulceration and regeneration of the mucosa and the polyploid mode of growth.

3. No true neoplasms were observed in any of the groups, although deep proliferations of epithelium occurred which could be mistaken for neoplasms unless studied in serial section.

4. Metaplastic bone formation in the flaps occurred, the result of chronic inflammation, but was not confined to the carcinogen-treated groups.

5. Implantation of threads into the flaps was not an effective means of introducing carcinogens, since the threads disappeared early in the experiments.

6. Simple painting of the flaps with carcinogens was deemed ineffective in view of the recurring ulceration of the mucosa. Experiments with different means of introducing carcinogens were considered necessary before the efficacy of the method in producing experimental adenocarcinoma of the stomach could be finally evaluated.

REFERENCES

1. CURTIS, M. R.; BULLOCK, F. D.; and DUNNING, W. F. A Statistical Study of the Occurrence of Spontaneous Tumors in a Large Colony of Rats. Am. J. Cancer, **15**:67–121, 1931.
2. DUNNING, W. F., and CURTIS, M. R. The Respective Roles of Longevity and Genetic Specificity in the Occurrence of Spontaneous Tumors in the Hybrids between Two Inbred Lines of Rats. Cancer Research, **6**:61–81, 1946.
3. HOWES, E. L., and DE OLIVEIRA, J. R. Early Changes in the Experimentally Produced Adenomas and Adenocarcinomas of the Stomach. Cancer Research, **8**:419–27, 1948.
4. KLEIN, A. J., and PALMER, W. L. Experimental Gastric Carcinoma. J. Nat. Cancer Inst., **1**:559–84, 1941.
5. LORENZ, E., and STEWART, H. L. Intestinal Carcinoma and Other Lesions in Mice Following Oral Administration of 1,2,5,6-Dibenzanthracene and 20-Methylcholanthrene. J. Nat. Cancer Inst., **1**:17–39, 1941.
6. ———. Squamous-Cell Carcinoma and Other Lesions of the Forestomach in Mice, Following Oral Administration of 20-Methylcholanthrene and 1,2,5,6-Dibenzanthracene. *Ibid.*, pp. 273–76.
7. OLCOTT, C. T. A Transplantable Nephroblastoma (Wilm's Tumor) and Other Spontaneous Tumors in a Colony of Rats. Cancer Resarch, **10**:625–28, 1950.
8. RATCLIFFE, H. L. Spontaneous Tumors in Two Colonies of Rats of the Wistar Institute of Anatomy and Biology. Am. J. Path., **16**:237–54, 1940.
9. SHAFFER, C. B.; CRITCHFIELD, F. H.; and MAIR, J. H., III. The Absorption and Excretion of a Liquid Polyethylene Glycol. J. Am. Pharm. Assoc., **39**:340–44, 1950.
10. ———. Chemical and Physical Examination of Some Polyethylene Glycols. *Ibid.*, pp. 344–48.
11. SHAY, H.; AEGERTER, E. A.; GRUENSTEIN, M.; and KOMAROV, S. A. Development of Adenocarcinoma of the Breast in the Wistar Rat Following the Gastric Installation of Methylcholanthrene. J. Nat. Cancer Inst., **10**:255–66, 1949.
12. SHAY, H.; GRUENSTEIN, M.; MARX, H. E.; and GLAZER, L. The Development of Lymphatic and Myelogenous Leukemia in Wistar Rats Following Gastric Installation of Methylcholanthrene. Cancer Research, **11**:29–34, 1951.
13. SMYTH, H. F.; CARPENTER, C. P.; and WEIL, C. S. The Toxicology of the Polyethylene Glycols. J. Am. Pharm. Assoc., **39**:349–54, 1950.
14. STEWART, H. L., and HARE, W. V. Variation in the Susceptibility of the Fundic and Pyloric Portions of the Glandular Stomach of the Rat to Induction of Neoplasia by 20-Methylcholanthrene. Acta Union internat. contre cancer, **7**:176–78, 1950.
15. STEWART, H. L.; HARE, W. V.; LORENZ, E.; and BENNETT, J. G. Adenocarcinoma and Other Lesions of the Glandular Stomach of Mice, Following the Intramural Injection of 20-Methylcholanthrene. J. Nat. Cancer Inst., **10**:359–60, 1949.
16. STEWART, H. L., and LORENZ, E. Induction of Adenocarcinoma of the Pyloric Stomach in Mice by Methylcholanthrene. J. Nat. Cancer Inst., **2**:193–96, 1942.
17. ———. Adenocarcinoma of the Pyloric Stomach and Other Gastric Neoplasms in Mice Induced with Carcinogenic Hydrocarbons. *Ibid.*, **3**:175–89, 1943.
18. ———. Morbid Anatomy, Histopathology and Histopathogenesis of Forestomach Carcinoma in Mice Fed Carcinogenic Hydrocarbons in Oil Emulsions. *Ibid.*, **10**: 147–66, 1949.
19. TOOVEY, E. W.; HELLER, L.; and WEBSTER, D. R. A Method of Transplanting Gastric Mucosa to the Anterior Abdominal Wall of the Rat for the Local Application of Carcinogens. Cancer Research, **11**:912–13, 1951.
20. VAN PROHASKA, J.; BRUNSCHWIG, A.; and WILSON, H. Oral Administration of Methylcholanthrene to Mice. Arch. Surg., **38**:328–33, 1939.
21. WELLS, H. G.; SLYE, M.; and HOLMES, H. F. Comparative Pathology of Cancer of the Alimentary Canal, with Report of Cases in Mice. Am. J. Cancer, **33**:223–38, 1938.

Studies on the Inherited Susceptibility and Inherited Hormonal Influence in the Genesis of Mammary Cancer in Mice*

JOHN J. BITTNER

(Division of Cancer Biology, Department of Physiology, University of Minnesota Medical School, Minneapolis 14, Minn.)

Additional observations will be presented in this report on the development of mammary cancer in mice, especially virgin females, associated with the action of an inherited hormonal influence, termed the inherited hormonal influence. Comparisons will also be made for the genic make-up of the inherited susceptibility versus the inherited hormonal influence.

While the role of the hormonal stimulation in the genesis of mammary cancer has been known for many years (see review by Loeb [26]), not until 1944 was it demonstrated that the genic control of the hormonal mechanism was related to the appearance of these tumors in virgin mice (15, 21). This was found by mating females of the A strain, where the incidence in virgin females was known to be low (6), with males of the Z or C3H stock, in which strain both the virgins and breeders have a high incidence. When the F_1 females were maintained as nonbreeders, a high incidence was noted. Thus, the hormonal mechanism which determined the high incidence in virgins of the Z strain was transmitted and called the inherited hormonal influence (9).

In this cross, the females of the A strain contributed the mammary tumor agent and the inherited susceptibility for mammary cancer, while the Z fathers transmitted the inherited hormonal influence, as well as the inherited susceptibility. The three primary factors (7) considered to be needed for a high incidence were represented.

Further studies from this laboratory indicated that in the A and Z cross (23, 28–30), as well as in other strains (10, 11, 13, 14, 23), the inherited hormonal influence was associated with postcastrational adrenal cortical hyperplasia, previously observed by Woolley *et al.* (33–35). Mice of strains which show adrenal lesions following gonadectomy have been tested for the transmission of hormonal mechanisms and their influence upon the development of mammary cancer.

* Assisted by grants from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service; the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council; and the Graduate School Cancer Research Fund of the University of Minnesota.

Received for publication May 5, 1952.

METHODS AND MATERIALS

The three strains of mice used in these studies were developed by Strong (31) and include the A, C3H, and I stocks. As in former reports, to simplify the hybrid designations, the C3H will be referred to as the "Z" stock.

The A and Z stocks were obtained from Strong more than 20 years ago and have been continued only by brother-to-sister matings. In both strains 65 successive generations of spontaneous mammary tumors have been observed.

After being used for various studies, including the reciprocal Z × I cross in co-operative work which showed the maternal influence on mammary cancer in mice (5, 25), the I line was discontinued. In 1944 representatives of the I stock were again obtained, this time from Andervont, who had observed (2, 3) that, when they receive the mammary tumor agent from C3H females, I mice were found to be relatively nonsusceptible to mammary cancer. However, following ovariectomy, females of the I strain developed adrenal cortical hyperplasia with hormonal stimulation of the secondary sex organs (23). This suggested that mice of the I stock might transmit the inherited hormonal influence, although they were "nonsusceptible" to mammary cancer.

To designate the hybrid crosses, the maternal stock is always given first. Thus, A females mated with I males produced AIF_1 hybrids. The F_2 hybrids resulted from mating the F_1 mice inter se. When the AIF_1 females were crossed with I males, the resulting back-cross animals were called AIF_1-IBC_1; the F_1 females mated with A males gave AIF_1-ABC_1 hybrids.

The AZF_1 (A♀ × Z♂) females were crossed with males of the A stock to produce the AZF_1-ABC_1 mice, and when the ABC_1 females were again back-crossed with A males, the next generation mice were called AZF_1-ABC_2 hybrids.

All the experimental animals received Purina Fox Chow and tap water. At least some animals of the various groups were observed during the same periods.

RESULTS

The observations on the occurrence of spontaneous mammary cancer are reported in Table 1 for breeding and virgin females of the various inbred stocks and their hybrids. The incidences were determined by including all mice which survived to the age at which the youngest mouse was observed to have mammary cancer. Also tabulated are the average ages for the appearance of tumors and the death of the noncancerous mice.

Eleven females of the I stock were foster-nursed by females of the Z or A cancerous strains, so they would obtain the mammary tumor agent, and later were maintained as breeders. All I mice of the fostered series and their 42 descendants of the following three generations failed to develop mammary cancer. Since breeders did not die cancerous, virgin females of the I stock with the agent were not observed.

Five females of the I stock of the fostered generation were mated with Zb (without the agent) males, and the incidence of mammary cancer was observed in their F_1 progeny. Among the nineteen hybrids with mothers obtaining the agent from A females, only one had cancer. Three I females, of one litter and nursed by a female of the Z stock, had 32 F_1 progeny. The incidences in the $IZbF_1$ progeny of these mothers were 10, 25, and 83 per cent. The average cancer age for those with the high incidence was 242 days; for the others it was 343 days.

The fostered females of the I stock had fifteen descendants of the first or second generation tested, by mating with susceptible, usually Zb, males. Two per cent of their 134 F_1 progeny developed cancer.

In the inbred A strain, the nonbreeding females gave the usual low incidence of mammary cancer (6), but the incidence increased to over 80 per cent when the females were subjected to the hormonal stimulation associated with the production of young and lactation. Virgins of the Z stock had a rather high incidence, and this was increased by breeding with an acceleration in the time of appearance (Table 1).

In the breeding AZF_1 and AZF_2 hybrids, as well as the back-cross generations to the A stock, approximately the same incidence was observed as in the inbred maternal A strain. With the exception of the AZF_1-ABC_2 generation, where only nineteen breeders were continued, the average cancer ages were also the same.

The percentage of AZF_1 virgins to have mammary cancer was greater than that found in the Z strain, or 89 and 66 per cent, respectively. In virgins of the AZF_2 generation, the incidence dropped to 62 per cent, with further reductions being noted in the first and second "A" back-cross generations.

At least seven of the AZF_1-ABC_2 progeny of fourteen ABC_1 mothers were continued in use as nonbreeders. These mothers had 151 offspring with the same incidence, 19 per cent, as the total number (Table 1); and they are listed, according to when the mothers either developed mammary cancer or died noncancerous, in Table 2, with data for their AZF_1-ABC_2 young.

One female died noncancerous, as did her nine AZF_1-ABC_2 progeny. Among the offspring of ABC_1 cancerous mothers, the incidence ranged from 0 per cent to 44 per cent. The average cancer ages could not be correlated with the incidence in the ABC_2 mice, although the highest incidence occurred in some at the earliest age. However, the average age of groups with incidences of 27 and 37 per cent, above the average, was over 600 days, or 4 months later than the entire group.

After the females of the A strain had been mated with I males, their AIF_1 hybrids, when main-

TABLE 1

DATA ON THE DEVELOPMENT OF MAMMARY CANCER IN INBRED MICE AND THEIR HYBRIDS

Stock or generation	Matings	Observations for breeding females				Observations for virgin females			
		No.	Per cent cancer	Av. age in days cancer	Av. age in days nonca.	No.	Per cent cancer	Av. age in days cancer	Av. age in days nonca.
A	A♀ × A♂	569	84	330	387	111	4	450	573
Z(C3H)	Z♀ × Z♂	858	95	278	322	153	66	404	499
I*	I♀ × I♂	53	0		463	0			
AZF_1	A♀ × Z♂	85	94	320	414	121	89	468	601
AZF_2	AZF_1♀ × AZF_1♂	116	83	337	367	153	62	486	683
AZF_1-ABC_1	AZF_1♀ × A♂	22	91	328	626	22	41	500	706
AZF_1-ABC_2	AZF_1-ABC_1♀ × A♂	19	89	389	549	159	19	498	640
AIF_1	A♀ × I♂	97	95	224	389	50	72	451	681
AIF_2	AIF_1♀ × AIF_1♂	82	68	302	549	155	30	469	676
AIF_1-ABC_1	AIF_1♀ × A♂	23	78	314	609	80	16	540	681
AIF_1-IBC_1	AIF_1♀ × I♂	25	56	274	631	84	44	482	715

* Eleven females of the I stock, fostered by Z or A females, and 42 descendants.

tained as breeders, had an incidence of 95 per cent; and in the succeeding generation, AIF_2, it was 68 per cent. Only small numbers of breeders were continued in the AIF_1-ABC_1 and AIF_1-IBC_1 generations; in the former the incidence was the same as in the maternal A strain, but in the IBC_1 breeders it was only 56 per cent. The shortest latent period required for the development of cancer was seen in the AIF_1 group; the next earliest average age occurred in the AIF_1-IBC_1 breeders.

Seventy-two per cent of the virgins of the AIF_1 generation had cancer. In the AIF_1-IBC_1 virgins a higher incidence (44 per cent) was found than in the AIF_2 mice (30 per cent); but the lowest incidence was observed in the AIF_1-ABC_1 virgins (Table 1).

In Tables 3–5 the pertinent data are listed, according to tri-monthly age periods, for virgins of all groups, except the AZF_1-ABC_1 hybrids. As only 22 mice were observed in this generation, they have not been included.

TABLE 2

OBSERVATIONS FOR MAMMARY CANCER IN VIRGIN AZF_1-ABC_2 HYBRIDS, LISTED ACCORDING TO MOTHERS WITH SEVEN OR MORE PROGENY

Cancer (+) or nonca. (−) age of AZF_1-ABC_1 mothers	No. of AZF_1-ABC_2 progeny	Incidence in progeny (per cent)	Av. ca. age in progeny
+257	8	13	460
+260	12	42	475
+272	7	14	553
+272	11	9	446
+292	14	7	571
+310	9	44	327
+320	9	11	442
+353	10	10	478
+362	7	0	
+375	15	27	629
+399	19	37	611
+517	10	10	571
+568	11	9	422
−623	9	0	

In Table 3 the number of mice to have mammary cancer or to die noncancerous is given by age periods. The percentage of the total number of virgins in each group to develop cancer during the various age periods has been tabulated in Table 4. The distribution would give a normal curve for every group except the AIF_1-ABC_1 mice, where, with only thirteen cancerous virgins, tumors appeared in three at an early age and in four after they were 21 months of age. The percentage of mice, living to the beginning of each age period or longer, to develop mammary cancer has been tabulated in Table 5.

DISCUSSION

Spontaneous mammary cancer will occur with a high incidence only in females with the mammary tumor agent, with an inherited susceptibility, and after an adequate hormonal stimulation of the mammary glands (7). In addition to the genetic susceptibility, the hormonal mechanism associated with its development in virgin females has also been found to be under genic control (15, 21) and has been termed the inherited hormonal influence (9).

TABLE 3

NO. VIRGIN FEMALES DYING DURING EACH AGE PERIOD (CANCER/NONCANCER)

Months	A	Z	AZF_1	AZF_2	AZF_1-ABC_2	AIF_1	AIF_2	AIF_1-ABC_1	AIF_1-IBC_1
−6	0/0	0/0	0/0	0/0	1/0	0/0	0/0	0/0	0/0
6–8	0/0	7/0	5/0	0/0	2/0	3/0	3/0	0/0	0/0
9–11	1/3	23/4	14/2	13/0	2/0	6/1	7/0	3/0	8/0
12–14	0/8	42/13	31/2	26/3	4/6	9/1	12/0	1/0	10/0
15–17	3/14	21/18	32/0	26/3	10/13	10/1	11/18	2/5	7/5
18–20	0/41	5/11	15/2	17/14	9/30	6/0	7/20	2/18	6/7
21–23	0/39	3/6	6/3	9/13	1/52	2/5	5/30	4/22	3/9
24–26	0/2	0/0	4/3	3/20	2/23	0/4	1/24	1/13	2/10
27–29+	0/0	0/0	1/1	1/5	0/4	0/2	0/17	0/9	1/16
No.	111	153	121	153	159	50	155	80	84
ca/nonca	4/107	101/52	108/13	95/58	31/128	36/14	46/109	13/67	37/47
Per cent cancer	3.6	66.0	89.3	62.1	19.5	72.0	29.7	16.3	44.0
Av. ca. age	450	404	468	486	498	451	469	540	482
Nonca. age	573	499	601	683	640	681	676	681	715

Our studies on hormonal mechanisms and their possible relationship with the genesis of mammary cancer were stimulated by the work of Woolley *et al.* (33–35), after it was observed that mice of certain inbred strains gave rise to postcastrational adrenal hyperplasia, followed by hormonal stimulation of the secondary sex organs, and, in a few instances, mammary cancer.

In these experiments (33–35), ovariectomized females of the dilute brown and C3H stocks (strains with a high incidence of mammary cancer in both virgin and breeding females) showed the characteristic adrenal alterations and hormone production. On the other hand, castrated females

of the C57BL stock did not, and neither was there evidence of hormonal stimulation. When breeders of this stock possessed the mammary tumor agent, either a low (2, 8) or high (3, 4, 18) incidence of mammary cancer was noted, depending upon the subline being tested, while nonbreeders had few tumors (27, 32).

That the hormonal mechanism was transmitted was determined by making reciprocal crosses between the A and Z stocks and finding a high incidence of mammary cancer in the virgin hybrids (15). Comparable findings were reported by Heston and Andervont (21) at approximately the same time. Further observations indicated that the inherited hormonal influence (9) and the inherited susceptibility for mammary cancer were not produced by the same genes, although common genes might exist (12–14).

Gonadectomized females of the A and Z stocks and their hybrids, with and without the agent, were also studied (28–30), and it was found that the groups which showed a high incidence of mammary cancer as virgins also had adrenal cortical hyperplasia. Mice of these groups without the mammary tumor agent also had the typical adrenal lesions but not mammary cancer. Castrated females of the A stock had slight adrenal changes but not hormonal production, since the accessory sex organs remained in the castrate state (17, 28–30).

Since these observations suggested the possibility that mice with the inherited hormonal capacity for developing mammary tumors as virgins might show postcastrational adrenal cortical hyperplasia, this theory has been tested in several crosses. To demonstrate this hormonal mechanism, females of the A strain were mated with males of the stock being tested. In several crosses, the high incidence of mammary cancer in virgin F_1 females showed that other strains (in addition to the Z strain) have been found to transmit the inherited hormonal influence (10–12). Stocks not previously reported include the C and I^s. Other hormonal mechanisms and their influence upon the development of mammary cancer have been discussed (10) and will be considered in future reports. In every cross, the inherited hormonal influence appears to be dominant over these other mechanisms.

As reported here, and in confirmation of the observations of Andervont (1, 3), females of the I stock are relatively nonsusceptible to mammary cancer, since they remained noncancerous after they obtained the agent and were maintained as breeders. However, ovariectomized females of the I stock developed adrenal cortical hyperplasia (23), suggesting that they might possess the inherited hormonal influence. This was proved by mating males of the I stock with females of the A strain and finding a high incidence of mammary tumors in the F_1 virgin females.

By observing the hybrids of the two crosses con-

TABLE 4

PERCENTAGE OF THE TOTAL OF EACH GROUP TO DEVELOP MAMMARY CANCER DURING EACH AGE PERIOD, WHEN THE FEMALES WERE MAINTAINED AS VIRGINS

Months	A	Z	AZF_1	AZF_2	AZF_1-ABC_2	AIF_1	AIF_2	AIF_1-ABC_1	AIF_1-IBC_1
–6	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0
6–8	0.0	4.6	4.1	0.0	1.3	6.0	1.9	0.0	0.0
9–11	0.9	15.0	11.6	8.5	1.3	12.0	4.5	3.8	9.5
12–14	0.0	27.5	25.6	17.0	2.5	18.0	7.7	1.3	11.9
15–17	2.7	13.7	26.4	17.0	6.3	20.0	7.1	2.5	8.3
18–20	0.0	3.3	12.4	11.1	5.7	12.0	4.5	2.5	7.1
21–23	0.0	2.0	5.0	5.9	0.6	4.0	3.2	5.0	3.6
24–26	0.0	0.0	3.3	2.0	1.3	0.0	0.6	1.3	2.4
27–29	0.0	0.0	0.8	0.7	0.0	0.0	0.0	0.0	1.2

TABLE 5

PERCENTAGE OF THE VIRGINS LIVING TO EACH AGE PERIOD, OR LONGER, TO DEVELOP MAMMARY CANCER

Months	A	Z	AZF_1	AZF_2	AZF_1-ABC_2	AIF_1	AIF_2	AIF_1-ABC_1	AIF_1-IBC_1
–6	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0
6–8	0.0	4.6	4.1	0.0	1.9	6.0	1.9	0.0	0.0
9–11	0.9	19.6	15.7	8.5	3.1	18.0	6.5	3.8	9.5
12–14	0.9	47.1	41.3	25.5	5.7	36.0	14.2	5.0	21.4
15–17	3.6	60.8	67.8	42.5	11.9	56.0	21.3	7.5	29.8
18–20	3.6	64.1	80.2	53.6	17.6	68.0	25.8	10.0	36.9
21–23	3.6	66.0	85.1	59.5	18.2	72.0	29.0	15.0	40.5
24–26	3.6	66.0	88.4	61.4	19.5	72.0	29.7	16.3	42.9
27–29	3.6	66.0	89.3	62.1	19.5	72.0	29.7	16.3	44.0

sidered here (A♀ × Z♂ and A♀ × I♂) as either virgins or breeders, it is possible to obtain information on the respective role of the inherited susceptibility and the inherited hormonal influence in the genesis of mammary cancer in mice. And, by considering the various groups, some comparisons may be made regarding the segregation of the genes controlling these inherited factors.

In the A♀ × Z♂ cross, the incidence of mammary tumors was as high in breeders of the four groups of hybrids (F_1, F_2, and two back-cross generations to the A strain) as was seen in breeders of the maternal strain. The lowest incidence, 83 per cent, was observed in the AZF_2 generation. Several of the noncancerous mice of this group died following alterations in the animal room, and this is reflected by the early age at death of the noncancerous mice, viz., 367 days—nearly 200 days before other series. The AZF_2 breeders of 24 litters, consisting of 73 mice, all had mammary cancer at an average age of 338 days.

From the data observed in these hybrids, it is possible to assume either that the mice of the A and Z stocks possessed the same susceptibility for spontaneous mammary cancer, or, if different genes were involved, that any combination of these genes would produce susceptible hybrids.

Eighty-nine per cent of the virgin AZF_1 females had mammary cancer, an increase of 23 per cent over that found in virgins of the Z strain. The incidence in the AZF_2 virgins of 62 per cent would indicate that the segregation of at least one primary gene was involved in controlling the inherited hormonal influence from the Z stock. Further evidence for this was apparent in the virgins of the AZF_1-ABC_1 and AZF_1-ABC_2 generations, where the incidences were 41 and 19 per cent, respectively.

The mothers of the AZF_1-ABC_2 generation have been tabulated, and only three had progeny with a total incidence of 40 per cent (Table 2). The average cancer age for mice of this group, 579 days, was later than that observed for the entire group. The incidence for the progeny of other AZF_1-ABC_1 mothers ranged from 0 to 27 per cent. Two females, one cancerous and one noncancerous, had no cancerous progeny. From these results, it appears probable that, while the ratio for the entire group might be explained on the segregation of a single gene, multiple factors determined the transmission of the inherited hormonal influence.

A breakdown of the AZF_1-ABC_2 data also revealed that the highest incidence was found in the progeny born to mothers which developed mammary cancer when they were from 300 to 400 days of age. When the mothers had tumors before they were 300 days of age, 17 per cent of their offspring developed cancer; and, when cancer did not appear in the mothers before 500 days, the incidence in their offspring was 10 per cent. No importance may be attached to these tabulations at present.

In the AIF_1 breeders, the same incidence of mammary cancer was observed as in the AZF_1 breeders; but the tumors appeared, on an average, 100 days earlier in mice of the former group. The incidence of 78 per cent in the AIF_1-ABC_1 breeders compares with the observations for the maternal stock, both as regards the incidence and average cancer age. Since the AIF_1-ABC_1 group consisted of only 23 mice, the incidence was influenced by the fact that three of the four mice in one litter died noncancerous. Five other females born to the same mother were also continued as breeders, and all had cancer; thirteen were kept as virgins, whose incidence was 28 per cent.

Breeders of the AIF_2 and AIF_1-IBC_1 generations possessed different susceptibilities for mammary cancer and had incidences of 68 and 56 per cent. Their tumors appeared slightly earlier than the average age observed in the A stock. Thus, at least one primary gene was indicated for the inherited susceptibility, with perhaps modifying genes acting to influence the average cancer age.

Although only 4 per cent of the virgins of the A stock and none of the breeders of the I stock with the mammary tumor agent had spontaneous mammary cancer, the cross between A females and I males produced hybrids with an incidence of 72 per cent, even when the females were observed as virgins. In this cross the mothers of the A stock contributed the inherited susceptibility for mammary cancer and the mammary tumor agent, while the inherited hormonal influence was transmitted by the I males.

Although the incidence of 72 per cent was lower than that observed in the AZF_1 virgins, it was comparable to that found in several groups with mothers of the same maternal strain (AD^2F_1, AD^8F_1, ACF_1) where the range was from 72 to 75 per cent (11, 13, and unpublished data).

The incidence of mammary cancer in virgins of the AIF_2 generation was determined by the segregation of the genes controlling both the inherited susceptibility and the hormonal influence. Based upon the observed incidence in the F_1 virgins, at least three genes were active—possibly more. The data for the AIF_1-ABC_1 virgins indicate that two or three genes controlled the inherited hormonal influence, and, in the AIF_1-IBC_1 group, one or two genes controlled the inherited susceptibility.

Females of the AZF_1-ABC_1 and AIF_1-ABC_1 generations would be expected to have approximately

the same proportion of chromatin from the A strain. The variation in the incidence of mammary cancer in breeders of the two groups was due primarily to three mice of one litter dying noncancerous in the latter series, referred to above. The incidence in the virgin females of the two generations would be influenced by the genes, transmitted by either the Z or I mice, which determine the inherited hormonal influence. In the virgins of the AZF_1-ABC_1 generation, 41 per cent had cancer, while the incidence in the AIF_1-ABC_1 group was only 16 per cent. On the basis of the incidence observed in the two virgin F_1 populations, the action of the inherited hormonal influence from the Z stock required at least one primary gene, while that from the I stock showed the action of multiple genes. Furthermore, the distribution of mammary tumors among the virgins of the AIF_1-ABC_1 generation was quite different from that observed in the other groups (Tables 3–5). This may have some significance, but the number of cancerous mice was not adequate for any interpretation.

From the observed incidence found in hybrids of these two crosses, and from comparisons with the theoretical ratios to be expected on a genetic basis, it is possible to summarize the data as follows:

Either the inherited susceptibility for spontaneous mammary cancer, transmitted by animals of the cancerous A and Z stocks, is controlled by the action of the same genes, or, if different genes are involved, any combination of these genes will produce susceptible hybrids in generations where segregation might be expected.

In the AI cross, the transmission of the inherited susceptibility from mice of the A strain may be explained upon the action of a single primary gene.

The incidence of mammary cancer in virgin females of the hybrid generations of the AZ cross may be interpreted according to the theory that the inherited hormonal influence of the Z stock is dependent upon one primary gene.

The inherited hormonal influence transmitted by mice of the I stock, resulting in mammary cancer in hybrids when maintained as virgins, involved the action of multiple genes.

The data suggest that the inherited hormonal mechanisms contributed by mice of the Z and I stocks may result from the action of not only different genes but also of different numbers of genes.

If it is possible that, in the Z stock, one or more genes common to both the inherited susceptibility and the inherited hormonal influence exists, then segregation of these genes could not be demonstrated in this study.

The genetic make-up of the two inherited factors considered in this report was based upon the theoretical relationship between the observed and expected ratios of cancer. As published previously (9), data on the transmission of the inherited susceptibility for mammary cancer by the A stock could not be explained by such a simple interpretation; it is more likely that multiple factors may be active to characterize both inherited factors under consideration.

One may also theorize regarding the difference in the average cancer ages observed in the hybrids, as compared to animals of the inbred strains. The hybrids continued as virgins had, in every generation, later average cancer ages than did those of the inbred Z strain; and yet the incidence in the AZF_1 and AIF_1 virgins was higher. The latent period for mammary cancer in the hybrids generally increased in proportion to the amount of chromatin they received from the A stock.

In the breeders of the AZ cross, the average cancer ages approximated more closely that seen in the A strain, whereas tumors in the AIF_1 breeders appeared 2 months earlier than in females of the Z strain. This early age was also observed in breeders of the AIF_1-IBC_1 group or in mice with increased chromatin from the I stock.

It may be suggested that either factors associated with the inherited hormonal influence or other inherited factors transmitted by mice of the I stock have some physiological effect which accelerates the time of development of mammary cancer in breeders of some generations.

Attempts have been made to investigate possible differences in the physiological nature of the hormonal patterns of mice with and without the inherited hormonal mechanism. Deringer, Heston, and Andervont (16) observed that the vaginas opened later in females of the A stock than in females of the C3H strain and their F_1 hybrids. There was also a difference in the initiation of estrus, but females of the A strain had cycles of shorter duration than did the C3H females.

By the transplantation of organs into either ovariectomized or adrenalectomized-gonadectomized F_1 hybrids, the grafted organs would be subjected to comparable or identical pituitary stimulation, and the hormones produced by the transplants would act upon and be metabolized by genetically identical tissues.

Ovaries from A, Z, and F_1 females were grafted into gonadectomized F_1 females, and the recipients were observed for many months (14, 23). The morphological difference, noted in intact animals, between the ovaries of the A and Z females was not evident after the ovaries had been maintained for 3 months in F_1 hybrids, in that the ovaries from

donors of the A strains were found to be indistinguishable from those of the Z or F_1 mice. This suggested a difference in pituitary function, and other findings indicated a variation in inherited hormonal properties of the ovaries of the parental strains, as well as other organs of the endocrine system. In another study (24), the change noted in adrenals following the transplantation into adrenalectomized-ovariectomized hybrids was due mainly to differences inherent in the adrenal tissue itself.

Other possible mechanisms by which inherited factors may operate, either the influencing of factors associated with the etiology of mammary cancer in mice or other physiological effects, have been suggested (17, 19, 20, 22).

These studies on the comparative action of the inherited susceptibility and the inherited hormonal influence indicate that other factors, in addition to the mammary tumor agent, are essential for the development of mammary cancer in mice. While some may be extrinsic in nature, others may be due to the action of genes and be inherited. A future report will be concerned with another hormonal effect which, although inherited, either inhibits or delays the time of appearance of mammary cancer, so that many animals die noncancerous in a group where a high incidence might be expected. Further basic research is needed to understand these mechanisms, since they may be fundamental to the problem of the genesis and control of cancer, at least in experimental animals.

SUMMARY

Further observations are presented on the action of another inherited factor, an inherited hormonal influence, which, along with the inherited susceptibility and the mammary tumor agent, has been found to be a primary cause of mammary cancer in virgin mice.

In several stocks there is an association between the presence of the inherited hormonal influence and the development of adrenal cortical hyperplasia in gonadectomized mice.

The inherited hormonal influence may be transmitted by mice of strains which are either susceptible or relatively nonsusceptible to the development of spontaneous mammary cancer.

The inherited susceptibility and the inherited hormonal influence, when transmitted by mice of the same stock, may be due to the action of different genes, although genes common to both factors may exist.

The inherited hormonal influence, when possessed by mice of different stocks, may not involve the same genic make-up in the two stocks.

The determination of the exact number of genes to produce either the inherited hormonal influence or the inherited susceptibility is difficult to ascertain because of the influence of other factors, intrinsic and/or extrinsic, and the source of the mammary tumor agent, in the development of mammary tumors. It seems probable that multiple genes are needed.

Possible physiological effects of the hormonal mechanism are discussed.

REFERENCES

1. Andervont, H. B. Further Studies on the Susceptibility of Hybrid Mice to Induced and Spontaneous Tumors. J. Nat. Cancer Inst., **1**:135–45, 1940.
2. ———. The Influence of Foster Nursing upon the Incidence of Spontaneous Mammary Cancer in Resistant and Susceptible Mice. *Ibid.*, pp. 147–53.
3. ———. Influence of Hybridization upon the Occurrence of Mammary Tumors in Mice. *Ibid.*, **3**:359–65, 1943.
4. Bagg, H. J. International Cancer Research Foundation, Report of Activities during 1939, p. 14.
5. Bittner, J. J. The Spontaneous Tumor Incidence in Mice. I. "Z" Stock × "I" Stock. J. Hered., **27**:391–93, 1936.
6. ———. Breast Cancer in Breeding and Virgin "A" and "B" Stock Female Mice and Their Hybrids. Pub. Health Rep., **54**:1113–18, 1939.
7. ———. "Influences" of Breast-Cancer Development in Mice. *Ibid.*, pp. 1590–97.
8. ———. Breast Cancer in Mice as Influenced by Nursing. J. Nat. Cancer Inst., **1**:155–68, 1940.
9. ———. Inciting Influences in the Etiology of Mammary Cancer in Mice. A.A.A.S., Research Conference on Cancer, pp. 63–95, 1945.
10. ———. Genetic Aspect of Cancer Research. Am. J. Med., **8**:218–28, 1950.
11. ———. Some Enigmas Associated with the Genesis of Mammary Cancer in Mice. Cancer Research, **8**:625–39, 1948.
12. Bittner, J. J., and Huseby, R. A. Relationship of the Inherited Susceptibility and the Inherited Hormonal Influence in the Development of Mammary Cancer in Mice. Cancer Research, **6**:235–39, 1946.
13. ———. Some Inherited Hormonal Factors Influencing Mammary Carcinogenesis in Virgin Mice. I. Genetic Studies, pp. 362–68. In E. S. Gordon (ed.), A Symposium on Steroid Hormones. Madison: Univ. Wis. Press, 1950.
14. ———. Some Inherited Hormonal Factors Influencing Mammary Carcinogenesis in Virgin Mice. II. A Study of Physiological Mechanisms. *Ibid.*, pp. 368–73.
15. Bittner, J. J.; Huseby, R. A.; Visscher, M. B.; Ball, Z. B.; and Smith, F. W. Mammary Cancer and Mammary Structure in Inbred Stocks of Mice and Their Hybrids. Science, **99**:83–85, 1944.
16. Deringer, M. K.; Heston, W. E.; and Andervont, H. B. Estrus in Virgin C3H (High-Tumor) and Virgin Strain A (Low-Tumor) Mice and the Reciprocal (A × C3H) F_1 Hybrids. J. Nat. Cancer Inst., **5**:403–6, 1945.
17. Dickie, M. M., and Woolley, G. W. Spontaneous Basophilic Tumors of the Pituitary Glands in Gonadectomized Mice. Cancer Research, **9**: 372–84, 1949.
18. Haagensen, C. D., and Randall, H. T. Milk-induced Mammary Carcinoma in Mice. Cancer Research, **5**:352–55, 1945.
19. Heston, W. E. Path of Gene Action in Mammary-Tumor Development in Mice. J. Nat. Cancer Inst., **7**:79–86, 1946.

20. ———. Role of Genes and Their Relationship to Extrachromosomal Factors in the Development of Mammary Gland Tumours in Mice. Brit. J. Cancer, **2**:87–90, 1948.
21. HESTON, W. E., and ANDERVONT, H. B. Importance of Genetic Influence on the Occurrence of Mammary Tumors in Virgin Female Mice. J. Nat. Cancer Inst., **4**:403–7, 1944.
22. HESTON, W. E.; DERINGER, M. K.; and ANDERVONT, H. B. Gene-Milk Agent Relationship in Mammary-Tumor Development. J. Nat. Cancer Inst., **5**:289–307, 1945.
23. HUSEBY, R. A., and BITTNER, J. J. Studies on the Inherited Hormonal Influence. Acta Union internat. contre cancer, **6**:197–205, 1948.
24. ———. Differences in Adrenal Responsiveness to Postcastrational Alteration as Evidenced by Transplanted Adrenal Tissue. Cancer Research, **11**:954–61, 1951.
25. JACKSON MEMORIAL LABORATORY, STAFF OF. The Existence of Non-chromosomal Influence in the Incidence of Mammary Tumors in Mice. Science, **78**:465–66, 1933.
26. LOEB, L. The Significance of Hormones in the Origin of Cancer. J. Nat. Cancer Inst., **1**:169–95, 1940.
27. MURRAY, W. S. Studies on the Effect of Foster Nursing and Its Relation to the Development of Mammary Carcinoma in the Mouse. Cancer Research, **1**:790–92, 1941.
28. SMITH, F. W. Castration Effects of the Inherited Hormonal Influence. Science, **101**:272–81, 1945.
29. ———. The Relationship of the Inherited Hormonal Influence to the Production of Adrenal Cortical Tumors by Castration. Cancer Research, **8**:641–51, 1948.
30. SMITH, F. W., and BITTNER, J. J. Castration Effects in Relation to the Inherited Hormonal Influence in Mice. Cancer Research, **5**:588, 1945.
31. STRONG, L. C. The Origin of Some Inbred Mice. Cancer Research, **2**:531–39, 1942.
32. VAN GULIK, P. J., and KORTEWEG, R. The Anatomy of the Mammary Gland in Mice with Regard to the Degree of Its Disposition for Cancer. Proc. Nederl. Akad. v. Wetenschappen, **43**:891–900, 1940.
33. WOOLLEY, G.; FEKETE, E.; and LITTLE, C. C. Mammary Tumor Development in Mice Ovariectomized at Birth. Proc. Nat. Acad. Sc., **25**:277–79, 1939.
34. ———. Difference between High and Low Breast Tumor Strains of Mice When Ovariectomized at Birth. Proc. Soc. Exper. Biol. & Med., **45**:796–98, 1940.
35. ———. Effect of Castration in the Dilute Brown Strain of Mice. Endrocinology, **28**:341–43, 1941.

Standardized Nomenclature for Inbred Strains of Mice

PREPARED BY

THE COMMITTEE ON STANDARDIZED NOMENCLATURE FOR INBRED STRAINS OF MICE

(T. C. CARTER, *Institute of Animal Genetics, Edinburgh, Scotland;* L. C. DUNN, *Columbia University, New York, N.Y.*; D. S. FALCONER, *Institute of Animal Genetics, Edinburgh, Scotland;* H. GRÜNEBERG, *University College, London, England;* W. E. HESTON, *National Cancer Institute, Bethesda, Maryland; and* G. D. SNELL, *Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine*)*

There are now in existence about 80 inbred strains of mice, some of which have been extensively used in research. Strains A, C3H, C57BL, and DBA, for example, have been employed in many laboratories for a variety of investigations including not only cancer research but also bacteriological, nutritional, endocrine, and other studies. Such wide dissemination of strains tends to result in the development of substrains. A number of sublines with known differences have thus arisen. Among the most familiar are sublines 1 and 2 of strain DBA and sublines a and cd of strain C57BR. Such sublines may differ in tumor incidence, in the frequency of certain congenital defects, in susceptibility to one another's transplantable tumors, or in other ways. Additional sublines have arisen through the occurrence in inbred strains of gene mutations affecting coat color or other visible characteristics, and still other lines have been artificially produced by the introduction of known genes into standard strains, or through the removal or introduction of the mammary tumor inciter by foster nursing or ova transfer.

This multiplicity of strains and substrains has created a need for a standard system of nomenclature. As a result of a communication to *Mouse News Letter* (1) in which this need was pointed out, a nomenclature committee consisting of T. C. Carter, L. C. Dunn, D. S. Falconer, H. Grüneberg, W. E. Heston, and G. D. Snell was organized. The rules for naming inbred strains and substrains, as finally developed, were submitted to the contributors to *Mouse News Letter*, together with a ballot which contributors were requested to mark and return. Thirty-four ballots were returned, of which 33 favored adoption of the rules and one was blank. Subsequently, the rules were somewhat further modified by the committee. The rules as finally approved are presented in Appendix 1.

Another problem considered by the committee was the extent to which the symbols for old and widely used strains should be changed to conform to the new rules. It was felt that certain minor changes might contribute to uniformity and would not cause serious confusion, provided they were generally accepted. Hence, the ballot sent to contributors to *Mouse News Letter* contained two alternative sets of symbols, the first providing maximum conformity to the proposed rules, the second minimum change from customary usage. It was stated in the accompanying letter that alternative 1 would be regarded as adopted only if approved by a two-thirds majority. The vote favored alternative 1 by 23 to 11, one more than the specified two-thirds.

In Appendix 2 is given a list of 124 inbred strains and substrains, with symbols conforming to the new, standardized usage. Most of the strains listed have been taken from *Mouse News Letter No. 5* (1); others have been gathered by a mail canvass conducted by members of the committee. The committee wishes to beg the indulgence of any persons maintaining inbred strains of mice who may have been overlooked.

Under each listed strain is given information, where available, as to synonyms of the preferred symbol, the number of generations of brother × sister inbreeding, genetic formula, origin, and characteristics. A list is also given for each strain, under the heading "Maintained by," of all persons or laboratories currently carrying the strain. The names of persons and laboratories are abbreviated, the abbreviations used being given in Appendix 3.

* Address requests for reprints to: Dr. G. D. Snell, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, or to The Laboratory Animals Bureau, Medical Research Council Laboratories, Holly Hill, Hamstead, London, N.W. 3., England.

These same abbreviations are intended for the designation of substrains in accordance with rule 4 in Appendix 1. Thus, the sublines of strain JK, maintained by Bu (Burdette) and St (Strong) are identified by the symbols JK/Bu and JK/St, respectively. Many of the abbreviations in Appendix 3 have already been listed in *Mouse News Letter No. 5* (1). It is urged that anyone raising inbred mice who is not included in this list send some suitable abbreviation for his name or the name of the laboratory at which he works to the editor of *Mouse News Letter* (1), so that the list can be revised as necessary and kept up to date.

The selection of substrains for inclusion in Appendix 2 has been based on (*a*) long separation and (*b*) the presence of known differences. In some cases the decision as to what strains should be given separate listing has necessarily been arbitrary. Many useful substrains differentiated from the parent strain by a single mutant gene have, for example, been omitted. As our knowledge of substrains increases, many additional ones will undoubtedly be found to have important individual characteristics. For a more complete listing of substrains the reader should consult *Mouse News Letter No. 5* (1).

The committee would like to urge that the full symbol, including substrain symbol, for strains listed in Appendix 2, be given in all published articles at least in the description of strains used. The extent to which substrain symbols should be used for substrains not individually listed is something that must be left to the individual investigator, but we wish to emphasize the need for the more general designation of substrains.

The information given as to the origin and characteristics of the different strains is necessarily brief. Further details will be found in five general references (2–6) and in references listed with each strain.

Because of inconsistencies in existing nomenclature, interpretation was sometimes necessary in transferring material from submitted strain lists to the list in Appendix 2. Wherever possible, doubtful cases were checked by correspondence. However, some errors may have been made. The committee requests that any inaccuracies be called to its attention. Corrections will be published in a subsequent issue of *Cancer Research*. Communications should be addressed to T. C. Carter or to G. D. Snell.

The members of the committee wish to express their deep appreciation to the numerous persons who have assisted by contributing lists of stocks and by providing information as to the origin and characteristics of various strains.

REFERENCES

1. Mouse News Letter is an informal mimeographed document carrying information on mutant genes and inbred strains in mice. The first issue was prepared by L. C. Dunn at Columbia University. Subsequent issues have been prepared by T. C. Carter and distributed by the Laboratory Animals Bureau, Medical Research Council Laboratories, Holly Hill, Hamstead, London, N.W. 3., Eng. It appears twice annually, and currently receives contributions from 30 laboratories.
2. Heston, W. E. Genetics of Mammary Tumors in Mice. A.A.A.S. Pub. 22, pp. 55–84, 1945.
3. ———. Genetics of Cancer. Adv. Genetics, 2:99–125, 1948.
4. ———. Development of Inbred Strains in the Mouse and Their Use in Cancer Research. R. B. Jackson Memorial Laboratory, 20th Commemoration Lectures, 1949.
5. Law, L. W. Mouse Genetics News No. 2. J. Hered., 39:300–308, 1948.
6. Strong, L. C. The Origin of Some Inbred Mice. Cancer Research, 2:531–39, 1942.

APPENDIX 1

RECOMMENDED RULES FOR SYMBOLS

To Designate Inbred Strains of Mice

1. *Definition of inbred strain.*—A strain shall be regarded as inbred when it has been mated brother × sister for twenty or more consecutive generations. Parent × offspring matings may be substituted for brother × sister matings, provided that in the case of consecutive parent × offspring matings the mating in each case is to the younger of the two parents.

2. *Symbols for inbred strains.*—Inbred strains shall be designated by a capital letter or letters in Roman type. It is urged that anyone naming a new stock consult Appendix 2 of this article or *Mouse News Letter* (1) to avoid duplication. Brief symbols are preferred.

An exception is allowed in the case of stocks already widely used and known by a designation which does not conform.

3. *Definition of substrain.*—The definition of substrain presents some of the same problems as the definition of species. In practice, the determination of whether two related strains should be treated as substrains, and whether, in published articles, substrain symbols should be added to the strain symbol, must rest with the investigators using them. The following rules, however, may be of help.

Any strains separated after eight to fifteen generations of brother × sister inbreeding and maintained thereafter in the same laboratory without intercrossing for a further fifteen to twenty generations shall be regarded as substrains. It shall also be considered that substrains have been constituted (*a*) if pairs from the parent strain (or substrain) are transferred to another investigator, or

(*b*) if detectable genetic differences become established.

4. *Designation of substrains.*—A substrain shall be known by the name of the parent strain followed by a slant line and the name of the person or laboratory maintaining it. Abbreviations with the initial letter set in Roman capitals may be substituted for the full name. Abbreviations should be brief, should as far as possible be standardized, and should be checked with published information or with some designated central agency to avoid duplication. Examples: A/He (Heston substrain of the A strain), A/Jax (Jackson Laboratory Inbred Nucleus substrain of the A strain).

Where a new substrain is created by transfer, the old substrain symbol shall be maintained and a new one added. Example: The A/He strain, on transfer from Heston to the Inbred Nucleus at the Jackson Laboratory, becomes A/HeJax. However, if a substrain symbol becomes cumbersome after several transfers, and if substrains with which it might be confused have been discontinued, it may be permissible to abbreviate it. Such abbreviations should be made a matter of public record in some suitable publication.

Where a substrain has known individuality and was originated by a person or laboratory no longer maintaining inbred stocks, it may be convenient to reduce the substrain symbol to one or two letters in lower case (as in C57BR/a and C57BR/cd).

When, within the hands of one investigator, new substrains arise by separation for fifteen or more generations, or by the occurrence of minor mutations, they shall be differentiated by numbers. (For sublines arising through the occurrence of a major mutation, i.e., a mutation to which a gene symbol has been assigned, see rule 5.)

5. *Isogenic stocks.*—Isogenic stocks produced by the occurrence of a single major mutation within an inbred strain or by the introduction of a marker gene into an inbred strain shall be designated by the strain symbol and substrain symbol (if any) followed by a hyphen and the gene symbol (in italics in printed articles). Example: CBA/Ca-*se*.

In the case of isogenic stocks produced by repeated crosses of a dominant gene into a standard inbred strain, it may be desirable to indicate the number of backcross generations. Example: C57BL/6-*W*^{v}(N8). The first hybrid or F_1 generation should be counted as generation 1, the first backcross generation as generation 2, etc.

6. *Substrains developed through foster nursing, ova transfer, or ovary transplant.*—Substrains developed by foster nursing shall be indicated by appending a subscript f to the strain symbol. Example: $C3H_f$. The strain used as foster parent may be indicated if desired by the addition of its symbol or an abbreviation for the same. Example: $C3H_fC57BL$ or $C3H_fB$ (C3H fostered on C57BL). In like manner strains developed through ova or egg transfer or ovary transplant shall be indicated by adding a subscript e or o, respectively. Example: A_eC57BL (A ova transferred to C57BL). When one investigator has several such substrains between which it is necessary to differentiate, the method described in the last paragraph of rule 4 is recommended.

7. *Indication of inbreeding.*—Where it is desired to indicate the number of generations of brother × sister inbreeding, this shall be done by appending, in parentheses, an F followed by the number of inbred generations. Example: A(F87). If, because of incomplete information, the number given represents only part of the total inbreeding, this should be indicated by preceding it with a question mark and plus sign. Example: YBL(F? + 10).

8. *Priority in strain symbols.*—If two inbred strains are assigned the same symbol, the symbol to be retained shall be determined by priority in publication. For this purpose, listing in *Mouse News Letter* shall be regarded as publication.

APPENDIX 2

List of inbred strains of mice and their major substrains, as recommended by the Committee on Inbred Strain Nomenclature.

Key to Abbreviations

Syn = synonyms of recommended symbol.
Inbr = number of generations of consecutive brother × sister inbreeding. Substrain on which figure is based is indicated in parentheses.
Gen = genetic constitution; only mutant genes are listed.
Orig = origin. In most cases a reference is given. Numbers in parentheses refer to references listed on p. 603. Where no reference is given, the information has usually been obtained from *Mouse News Letter* (1) or by correspondence.
Charac = characteristics. References same as under "Orig." "Jax" refers to data from the Inbred Nucleus of the Roscoe B. Jackson Memorial Laboratory.
Maintained by: persons or laboratories maintaining each strain are indicated by the abbreviations listed in "Appendix 3."
b × s = brother × sister.
* Not now being maintained by brother × sister matings.

A Inbr (Bs): 87. Gen: *aabbcc*. Orig: Strong, from cross made in 1921 of albino from Cold Spring Harbor and Bagg albino (6). The majority of sublines trace to a stock which Bittner obtained from Strong in 1927. Charac: mammary tumor incidence high in breeders, low in virgins (*Cancer Research*, **1**:113, 1941). Lung tumors, high in breeders and in virgins (*J. Nat. Cancer Inst.*, **3**:79, 1942). Produces 5–10 per cent young with cleft palate. High incidence of renal disease in old mice (*J. Nat. Cancer Inst.*, **5**:17, 1944–45). Maintained by: A, Am, Br, Bs, Bu, Cr, Dm, Fa, Fr, G, Gd, Go, Gr, Ha, Ka, Ki, Mg, Mi, Mo, Or, Pa, Rl, Si, Sn, Sp, St, Vi, Wa, Wo, Wy.

A/He Inbr (Jax): 87. Gen: *aabbcc*. Orig: Strong 1921, to Bittner 1927, to Heston 1938, to Jax 1948 at F77. Charac: mammary tumor about 80 per cent in breeding females (Jax, unpublished data). Maintained by: Fe, He, Hu, Jax, N, Rr.

A/Jax Inbr (Jax): 82. Gen: *aabbcc*. Orig: Strong 1921, to Cloudman 1928, to Jax 1948 at F73. Charac: mammary tumor about 20 per cent (Jax, unpublished data). Lower per cent granulocytes than other A sublines (*Proc. Soc. Exper. Biol. & Med.*, **78**:761, 1951). Maintained by: Jax.

A/L Syn: A subline Lilly. Inbr (N): 87. Gen: *aabbcc*. Orig: Strong 1921, to Bittner 1927, to W. Murray, to E. Lilly and Co. 1941, to Jay 1948, to Laboratory Aids Branch, National Institutes of Health, 1951. Maintained by: Ks, L, N.

A_fB Orig: A fostered on C57BL in 1948. Maintained by: A.

AB Syn: Albino-Bluhm. Inbr. (Hg): 45+. Gen: albino. Orig: Bluhm. Maintained by: Hg, Ro.

ABC *See* CBA_f. Note also that ABC has been used for a mixed strain produced by crossing an F_1 hybrid, usually C57BL × A, to strain A (ABC = A backcross). The mice so produced are genetically heterogeneous, but because they have one A parent will grow strain A tumors.

ADW Inbr (Rr): 20. Gen: *bb?ccDwdw*. Orig: MacDowell. Maintained by: Rr.

Afb *See* AKR.

AK/a Syn: Aka. Inbr: 69+. Orig: Furth, 1928, to Engelbreth-Holm in 1936 at F20+. Maintained by: Eh.

AK/Gl Orig: Gorer, to Haddow, to Glücksmann. Prior history undetermined. Maintained by: Gl.

AK/n Syn: Ak-n. Inbr: 18 gen by Gs. Orig: Furth, to Gross in 1945. Maintained by: Gs.

AKR Syn: Ak, Akm, Rockefeller Institute Leukemia, Afb, R.I.L., RIL. Inbr (Jax): 31. Gen: *aacc*. Orig: Carried by Furth as high leukemia strain from 1928 to 1936. Then randombred for several generations at Rockefeller Inst. followed by 8–9 generations of inbreeding by Mrs. Rhoads and 30+ by C. Lynch (*Cancer*, **1**:399, 1948). Charac: high leukemia (*Cancer Research*, **4**:622, 1944; *J. Gerontol.*, **1**:46, 1946). Maintained by: Du, Fe, Fu, Jax, Lw, Ly, M, Pa, Sk, Wy.

B *See* C57BL.

Ba *See* BALB/Gw

Bagg or Bagg albino *See* BALB, BL.

Bagg L *See* BL.

BALB Syn: B alb, Bagg albino, CSH Bagg Albino. Inbr (Jax): 82. Gen: *bbcc*. Orig: albino stock acquired by Bagg in 1906, to Little, to MacDowell in 1922; b × s inbreeding started by MacDowell in 1923. Prior inbreeding uncertain (*J. Gen. Physiol.*, **11**: 57, 1927–28, and correspondence). Maintained by: Co, Jax, M, Md, T.

BALB/c Syn: B alb C, C. Inbr (Jax): 51. Gen: *bbcc*. Orig: subline of BALB. Transferred from MacDowell to Snell in 1932 at F26 and subsequently widely distributed. Charac: low mammary tumor, but gives high incidence of mammary tumors when milk agent is introduced (*J. Nat. Cancer Inst.*, **1**:147, 1940–41). Susceptible to chronic pneumonia. Maintained by: Di, Fe, Fs, Gn, Go, Hu, Jax, Ki, Mg, Mn, N, Rl, Rr, Sc, Sn, Sp.

BALB/Ci Syn: BALB/Lafayette. Gen: *bbcc*. Orig: subline of BALB. Transferred from MacDowell to Caspari in 1934. Maintained by: Ci.

BALB/Gw Syn: Ba. Inbr (Gw): ? + 41. Gen: *bbcc*. Orig: MacDowell, to Gowen in

1932 at F27 (*J. Infect. Dis.*, 71:131, 1942). Charac: low resistance to *Salmonella typhimurium* (J. Infect. Dis., 77:85, 1945). Maintained by: Gw.

BAMA Orig: cross of A ♀ × MA ♂. Charac: low tumor. Maintained by: Sp.

BCHP Inbr (Re): 30+. Gen: *bbc^{ch}p/c^{ch}p*. Orig: Gates. Charac: vigorous stock. Maintained by: Re.

BDP Inbr (Jax): 33+. Gen: *aabbdse/dsepprr*. Orig: Gates. Maintained by: Re.

BL Syn: Bagg L, BALB/R. Inbr (Ly): 46. Gen: *aabbcc*. Orig: Lynch, from Bagg stock via Strong, but maintained at Rockefeller Inst. as distinct strain since 1921. Some of the original animals obtained from Strong carried agouti (5). Charac: mammary tumors 27 per cent in breeding ♀ ♀ and 4 per cent in virgins over 6 mo.; lung tumors 37 per cent in old mice (*J. Exper. Med.*, 43: 339, 1926). Maintained by: He, Ly.

BLCP Syn: BLcp. Gen: *aac^{ch}c^{ch}ss*. Orig: Strong. Maintained by: N.

BRS Syn: BrS, Br-S, Br-s. Inbr (N): 28. Gen: *aabb*. Orig: Strong, from a branch of strain NH treated for 8 or more generations with methylcholanthrene (*J. Nat. Cancer Inst.*, 5: 339, 1944–45). Charac: high gastric tumor (*Cancer Research*, 11:94, 1951). Maintained by: N. *See Also* 3CAMG.

BRSUNT Syn: BrSunt. Inbr (N): 37. Orig: Strong, a branch of BRS continued without further methylcholanthrene treatment. UNT = untreated. Maintained by: N, St.

BTRU Inbr (Co): 20. Gen: *a^{t}a^{t}ruru* anophthalmic. Orig: Dunn and Gluecksohn-Waelsch. Maintained by: Co.

BTWB Syn: BTwb. Inbr (Jax): 36. Gen: *a^{t}a^t*. Orig: Dunn, to Jax in 1948 at F28. Maintained by: Jax.

BUA Inbr (Wi): 15. Orig: Wilson. Charac: selected for growth and reproductive performance. Maintained by: Wi.

BUB Inbr (Wi): 18. Orig: Wilson. Charac: selected for growth and reproductive performance. Maintained by: Wi.

BUC Inbr (Wi): 14. Orig: Wilson. Charac: selected for growth and reproductive performance. Maintained by: Wi.

BUD Inbr (Wi): 18. Orig: Wilson, separated from BUB at 6th generation. Charac: selected for circling. Maintained by: Wi.

C Inbr (St): 60. Gen: *bb*. Orig: *see* C3H. Charac: tendency to bifurcation of seminal vesicles (*Anat. Rec.*, 81:333, 1941). Maintained by: St.

C *See* BALB/c.

CBA Syn: XXXIX. Inbr (Jax): 77. Gen: +. Orig: Strong (*See* C3H). Charac: mammary tumor incidence variously reported 1.1–22.2 per cent (2). Some hepatomas (4). Maintained by: A, Br, Ca, Fa, G, Ge, Gn, Gr, Jax, Ka, Ki, La, Mi, Mo, Mr, No, Pu, Sn, St, Wy.

CBA$_t$ Syn: ABC, CBA$_t$C3H. Orig: Paterson, from CBA mice received in 1949 from Carr and fostered on C3H. Charac: high mammary tumor. Maintained by: Ge*, Pa.

CBAN Inbr (St): 30. Gen: *aass*. Orig: Strong, from CBA × N. Maintained by: St.

CBAN-*p^r* Gen: *aasspp^{r}p^r*. Orig: Strong, spontaneous mutant. Maintained by: St.

CDE Syn: cdE, DE. Inbr (Wy): 14. Gen: *c^{e}c^e*. Orig: Eaton, to Woolley in 1948. Maintained by: Wy.

CE Syn: extreme dilution, ce. Inbr (Jax): 43+. Gen: *c^{e}c^e*. Orig: Woolley. Charac: low tumor, wide range of tumor types, high adrenal cortical carcinoma when ovariectomized, at birth (*Cancer Research*, 5:193, 1945). Maintained by: Jax, T, Vi, Wy.

CFCW Gen: *ccCaCa*. Maintained by: Rl.

CFW Gen: *cc*. Orig: Webster. Maintained by: Rl.

CHI Inbr (St): 65. Gen: +. Orig: Strong (*see* C3H). Maintained by: St.

CHI-*ps* Gen: *ppss*. Orig: Strong, methylcholanthrene treatment. Maintained by: St.

CHI-*s* Gen: *ss*. Orig: Strong, methylcholanthrene treatment. Maintained by: St.

CSH Bagg Albino *See* BALB.

C3H Syn: Z. Inbr (Jax): 58+. Gen: +. Orig: Strong, 1920, from cross of ♀ Bagg albino × ♂DBA. Strains C,

CBA, CHI, and HE originated from this same cross (6; *Genetics*, **20**:586, 1935). Charac: mammary tumor incidence high in breeding and virgin ♀♀ (*Cancer Research*, **1**:115, 1941). Many hepatomas (*Acta Union internat. contre Cancer*, **7**:616, 1951). Low red and white cell counts (*Proc. Soc. Exper. Biol. & Med.*, **78**:761, 1951). Maintained by: A, Am, Bs, Bu, Cr, Di, Dm, Du, Eh, Fe, Fr, Fs, Ge*, Gl, Gn, Go, Gs, Ha, He, How, Hu, Jax, Mi, N, Pa, Pu, Rl, Rr, Ru, Sc, Sp, St, Tn, Vi, Wa, Wi, Wo, Wy.

C3H/Ks — Syn: C3H/Jax, but the line now called C3H/Jax is one obtained from Heston. Orig: from C3H mice of unknown pedigree which survived the Bar Harbor fire. Charac: resistant to some C3H transplantable tumors. Maintained by: Jax, Ks.

$C3H_e$/JFe — Inbr (Fe): 7 since egg transfer. Orig: from C3H ova transferred to C57BL/6. Charac: low mammary tumor. Maintained by: Am, Fe.

$C3H_f$/He — Syn: $C3H_b$, $C3H_fB$. Inbr (He): 14 since fostering. Orig: Heston, from litter of C3H born by Caesarean section and foster-nursed on strain C57BL, 1945. Charac: low mammary tumor. Maintained by: Du, He, N. Other foster-nursed lines are maintained by: Fe, Gs, Ha, Lw, Wy.

C12I — *See* HE.

C57BL — Syn: C57 black, B. Inbr (He): 41+. Gen: *aa*. Orig: Little, 1921, from heterozygous sib pair, ♀57 × ♂52, from Miss Lathrop's stock. ♂52 mated to littermate ♀58 gave rise to strain C58. *See also* C57BR/a, C57BR/cd and C57L. Charac: mammary tumors about 1 per cent in bred ♀ ♀, 0 in virgins (2). Various types of internal tumors, mostly lymphosarcoma, 10–20 per cent (*Am. J. Cancer*, **36**:431, 1939). Eye abnormalities in about 10 per cent of animals, but incidence varying considerably in different sublines; hydrocephalus appears in some sublines. Maintained by: A, Ci, Cr, Dm, Du, Fa, Fr, G, Gl, Go, Gr, Gs, Ha, He, How, Ka, Ki, Mi, Mn, Mo, Or, Rl, Sp, St, T, Tn, Vi, Wa, Wo. *See also* $C57BL_{bc}$.

C57BL-a^t — Inbr (Jax): 30+. Gen: a^ta^t. Orig: mutation in C57BL. Maintained by: Jax, T.

C57BL-*b* — Inbr (Jax): 33. Gen: *aabb*. Orig: mutation in Warner's C57BL in 1937. Maintained by: Jax, Wa.

$C57BL_{bc}$ — Inbr (A): 23. Orig: Kortweg, DBA female backcrossed ×♂ C57BL 20 times, then b×s mating. Maintained by: A.

C57BL/6 — Syn: C57 black subline 6, B/6. Inbr (Jax): 32+. Gen: *aa*. Orig: separated from C57BL/10 prior to 1937. Charac: differs from C57BL/10 in incidence of eye defects. Maintained by: Bs, Ch, Fe, Hu, Jax, N, Re, Rr, Si, Sn, T.

C57BL/6Ks — Charac: resistant to some C57BL/6 transplantable tumors. Maintained by: Ks.

C57BL/10 — Syn: C57 black subline 10, B/10. Inbr (Jax): 41+. Gen: *aa*. Orig: separated from C57BL/6 prior to 1937. Charac: differs from C57BL/6 in incidence of eye defects. Maintained by: Bs, Ch, Fs, Gn, Jax, Rr, Sc, Sn, Wy.

C57BR — Syn: C57 brown, BR. Gen: *aabb*. Orig: *See* C57BR/a *and* C57BR/cd; all existing branches probably belong to one or the other of these sublines. Maintained by: Rl, Sp, T.

C57BR/a — Syn: C57 brown subline a, BR/a. Inbr (Jax): 78. Gen: *aabb*. Orig: Little, from same ♀57 and ♂52 that gave rise to C57BL (which see), C57BR/cd, and C57L. Black and brown lines were separated in the first generation. C57BR/a was separated from other brown lines in the 9th generation. Charac: low mammary tumor (4). Maintained by: Jax, Sn, T, Wy.

C57BR/cd — Syn: C57 brown subline cd, BR/cd. Inbr (Jax): 76. Gen: *aabb*. Orig: Little, from same ♀57 and ♂52 that gave rise to C57BL, C57BR/a, and C57L. Black and brown lines were separated in the first generation. Subline cd was established in generation 13 from a cross of two brown branches, one of which had previously given rise to line C57BR/a. Charac: low mammary tumor (4). Maintained by: He, Jax, N.

C57L — Syn: C57 leaden, leaden, M, L, LN. Inbr (Jax): 53. Gen: *aabblnln*. Orig:

J. Murray, 1933, mutation in generation 22 of a C57BR subline of which the nonleaden (brown) branch is now extinct. Charac: low mammary tumor (2). Maintained by: He, Jax, N, Rl, T, Vi.

C58 Inbr (Jax): 87. Gen: *aa*. Orig: MacDowell, from mating made in 1921 of littermates ♀58 and ♂5 of the Lathrop stock. ♂52 mated to littermate ♀57 gave rise to strains C57BL, C57L, C57BR/a, and C57BR/cd. Charac: high leukemia (*Proc. Soc. Exper. Biol. & Med.*, **68**: 571, 1948). Maintained by: Fe, Ha, Hu, Jax, Ki, Lw, M, Md, N, Si, Wy.

C58-a^t Orig: Law, a^t gene introduced at F79, then backcrossed to C58 for 7 generations. Maintained by: Lw.

D *See* DBA.

DBA Syn: dilute brown, dba, dbr, D. Inbr (Eh): 58+. Gen: *aabbdd*. Orig: Little, in 1909. Charac: high mammary tumor in bred ♀♀, variously reported as medium to high in virgins (2). Maintained by: A, Eh, Ha, Hg, Jax, Rl, Sp, Tn.

DBA_f Syn: dba_b. Orig: sublines of DBA fostered on C57BL. Maintained by: A, Ki.

DBA/Wa Inbr (Jax): 31+. Orig: Little, to W. Murray, to Warner. Maintained by: Jax, Wa.

DBA/1 Syn: dba subline 1, dba-1, D/1. Inbr (Fe): 32+. Gen: *aabbdd*. Orig: Little, 1909. In 1929–30 some crosses were made between sublines and several new sublines established. Two of these were 12 (now called 1) and 212 (now called 2). Charac: resistant to DBA/2 transplantable tumors. Maintained by: Fe, Gn, Hu, Jax, Si.

DBA/2 Syn: dba subline 2, dba subline 212, dba-2, D/2. Inbr (Jax): 37+. Gen: *aabbdd*. Orig: *see* DBA/1. Charac: 30–40 per cent leukemia (3). Resistant to DBA/1 transplantable tumors. Maintained by: Bs, Fs, Gn, Jax, Ki, Lw, Mi, N, Rr, Vi, Wy.

$DBA/2_f$ Orig: Woolley, DBA/2 fostered on C57BL. Maintained by: Wy. Another line is maintained by Lw.

dbr *See* DBA.

dilute brown *See* DBA.

E Inbr (Gw): 37. Gen: *cc*. Orig: Gowen, from dealer stock in Pennsylvania. Charac: medium resistance to *Salmonella typhimurium* (*J. Infect. Dis.*, **77**:85, 1945). Maintained by: Gw.

F Inbr (St.): 55. Gen: *aabbc^{ch}c^{ch}ddss*. Orig: Strong (6). Charac: about 50 per cent leukemia (*Proc. Soc. Exper. Biol. & Med.*, **55**:147, 1944). Maintained by: St.

FA Inbr (Ki): 36. Gen: *aabbc^{ch}c^{ch}ddss*. Orig: Kirschbaum, cross of F × A, followed by two backcrosses to strain F. Charac: high leukemia. Maintained by: Ki.

FB Inbr (Ki): 26. Orig: Kirschbaum, cross of F × A, followed by backcross to strain F. Charac: moderate leukemia. Maintained by: Ki.

FUCFW Inbr (Co): 21. Gen: *ccFuFu*. Orig: Dunn and Gluecksohn-Waelsch. Maintained by: Co.

GFF Inbr (G): 25. Gen: *aabbpp*. Orig: Glaxo Labs. Maintained by: G.

HE Syn: C12I. Inbr (St): 70. Gen: *dd*. Orig: Strong (*see* C3H). Maintained by: St.

I Inbr (St): 50. Gen: *aabbddppss*. Orig: Strong (6). Maintained by: Sp, St.

I-c^{ch} Inbr (Jax): 60. Gen: *aabbddc^{ch}p/c^{ch}pss*. Orig: Subline of I. Andervont obtained from J. Murray in 1935 at F22. Origin of c^{ch} in this subline is unknown. Maintained by: Jax, T.

IF Inbr (Br): 40. Gen: a^ta^t. Orig: Bonser, by selection of mice which developed papillomas early following application of carcinogens. Charac: mammary tissue very sensitive to carcinogenic agents, either distant or local. Very low spontaneous mammary cancer incidence in virgin ♀♀. Maintained by: A, Br, Or, Wo.

JK Inbr (St): 50. Gen: *aabbppsese*. Orig: Strong, 1927 (6). Charac: low cancer (*J. Hered.*, **28**:40, 1937). Maintained by: Bu, St.

KICF Inbr (Co): 22. Gen: *ccKiki*. Orig: Dunn and Gluecksohn-Waelsch. Maintained by: Co.

Kreyberg albino or Kreyberg white label *See* WHL/Br.

L Inbr (St): 50. Gen: $A^wA^wc^{ch}c^{ch}$. Orig: Strong (6). Charac: low mam-

mary tumor, some lymphoblastoma (*Am. J. Cancer*, **20**:72, 1934). Maintained by: St.

L — *See* C57L. *See* LGW. *See* 50.

LII — *See* PL.

leaden — *See* C57L.

LGW — Syn: L. Inbr (Gw): 38. Gen: *aasisi*. Orig: "silver" strain from English fancier, to Dunn, to MacDowell, to Iowa State. Inbred at Iowa State (*J. Infect. Dis.*, **71**:131, 1942). Charac: susceptible to *Salmonella typhimurium* (*J. Infect. Dis.*, **77**:85, 1945). Maintained by: Gw.

LP — Syn: Line 11 pied, LINE 11. Inbr (Jax): 37. Gen: A^wA^wss. Orig: Dunn. Maintained by: Di, Jax.

M — *See* C57L.

MA — Syn: Marsh, Marsh albino. Old Buffalo and New Buffalo were branches of strain MA. Inbr (Jax): 31+. Gen: *cc*. Orig: Marsh's strain 3. Started from pair of mice from Lathrop-Loeb colony. Thirty-two gens. mostly random mating by Marsh, to W. Murray who started b × s matings, to Warner (*J. Cancer Research*, **13**:313, 1929; *Am. J. Cancer*, **34**:434, 1938). Charac: high mammary tumor (2). Maintained by: Jax, Sp, Wa.

MA/My — Syn: ND. Inbr (Jax): 28+. Gen: *cc*. Orig: low tumor line segregated from strain MA by W. Murray and Warner after 37 b × s generations (*J. Nat. Cancer Inst.*, **7**:183, 1946–47). Maintained by: Jax, Sp, Wa.

MABA — Orig: cross of MA ♀ × A ♂. Charac: low tumor. Maintained by: Sp.

N — Inbr (St): 45. Gen: *aabbddss*. Orig: Strong (6). Charac: low tumor incidence (*Am. J. Cancer*, **39**:347, 1940). Maintained by: St, Vi.

NB — Inbr (Re): 41+. Gen: *aabbc^{ch}p/c^{ch}pdse/dse*. Orig: Green. Maintained by: Gn, Lw, Re, Rl.

ND — *See* MA/My.

NH — Inbr (Wy): 22. Gen: *ddppss*. Orig: Strong, from cross involving strains CBA, N, and JK, to Kirschbaum, to Woolley (*Am. J. Cancer*, **39**:347, 1940). Charac: low tumor incidence, but 30–42 per cent lung tumors in very old mice (*J. Nat. Cancer Inst.*, **5**:339, 1944–45). Maintained by: Ki, Lw, Wy.

O20 — Inbr (A): 86. Orig: Kortweg. Maintained by: A.

P — Inbr (Sn): 47. Gen: *aabbdse/dsepprr*. Orig: Snell, extracted following outcross of similar stock developed by Gates. Maintained by: Ca, Gn, Jax, Mn, Sn.

PBR — Syn: pBr. Inbr (St): 30. Gen: *aabbpp*. Orig: Strong (*J. Gerontol.* **6**:340, 1951). Maintained by: St.

PL — Syn: PL(B), Princeton leukemia, LII. Inbr (Ly): 22. Gen: albino. Orig: from noninbred "Princeton" strain started in 1922 from 200 mice purchased from dealer (*J. Infect. Dis.* **82**:169, 1948). Inbred by Lynch, giving rise to a high leukemia branch B (= PL) and a second branch A (= PLA) with lower tumor incidence. Charac (Ly): low mammary tumor, 80–90 per cent leukemia. Maintained by: Du, Jax, Lw, Ly, M.

PLA — Gen: albino. Orig: *See* PL. Maintained by: Ly.

RF — Syn: Rf, Rfa. Gen: albino. Orig: Furth, 1928, from unknown stock of the Rockefeller Inst. Charac: low leukemia (*Am. J. Cancer*, **19**:521, 1933; *Cancer Research*, **1**:957, 1941). Maintained by: Fu.

RI — Inbr (Gw): 31. Gen: *aabbcc*. Orig: Gowen, from Webster's resistant stock in 1936, inbred b × s since that time (*J. Infect. Dis.*, **71**:131, 1942). Maintained by: Gw.

RIET — Inbr (A): 31. Orig: Kortweg. Maintained by: A.

R.I.L. — *See* AKR.

RIII — Syn: Paris, R3. Inbr (Br): 28. Gen: *cc*. Orig: Dobrovolskaïa-Zavdskaïa. Charac: high mammary tumor (*Arch. Path.*, **33**:411, 1942). Maintained by: Br, Ch, Cr, Dm, Fa, La.

RIII/Jax — Inbr (Jax): 31+. Gen: *cc*. Orig: Dobrovolskaïa-Zavdskaïa, to Wood, to Eisen, to Jax 1948. Charac: resistant to RIII/Wy transplantable tumors. Maintained by: Fe, Hu, Jax.

RIII/La — Orig: original Dobrovolskaïa-Zavdskaïa line. Charac: low mammary tumor. Maintained by: La.

RIII/Wy — Inbr (Wy): 33. Gen: *cc*. Orig: Dobrovolskaïa-Zavdskaïa, via Curtis and Dunning. Maintained by: Wy.

RIII$_f$B — Syn: RIIIX. Orig: Pullinger, RIII

fostered on C57BL. Maintained by: Dm, Pu.

S Inbr (Gw): 47. Gen: *cc*. Orig: Schott, obtained from dealer (Schwing) in 1927 (Genetics, **22**: 264, 1937). Subsequently selected for resistance to *Salmonella typhimurium* and inbred b × s by Gowen (*Ann. Rev. Microbiology*, 1948, p. 215). Maintained by: Gw.

SEA-*se* Syn: Sese-Ab. Inbr (Gn): 31+. Gen: *bbdSe/dse*. Orig: Green. Maintained by: Gn, Mn, Rl.

SEC-*dse* Syn: Sese-C. Inbr (Gn): 26+. Gen: *aabbc^{ch}c^{ch}DSe/dse*. Orig: Green. Maintained by: Gn, Mn, Re, Rl.

SEC/1-*se* Syn: Sese-C1. Inbr (Gn): 26+. Gen: *aabbc^{ch}c^{ch}Sese*. Orig: Green. Maintained by: Gn.

SEC/2-*d* Syn: Sese-C2. Inbr (Gn): 26+. Gen: *aabbc^{ch}c^{ch}Dse/dse*. Orig: Green. Maintained by: Gn.

ST Syn: Street. Inbr (Eh): 36. Gen: *aabbcc*. Orig: Engelbreth-Holm. Charac: 1–2 per cent leukemia including some plasma cell leukemias. Other tumors, principally pulmonary adenomas and mammary carcinomas, about 3 per cent (*Acta Union internat. contre cancer*, **7**:701, 1951). Maintained by: Eh, Jax, Ks.

STOLI Syn: StoLi, Storrs-Little. Inbr (Wy): 78. Gen: *aabbddpp*. Orig: MacDowell, from cross involving DBA and Dunn piebald (4). Charac: low leukemia (*Cancer Research*, **5**:65, 1945). Maintained by: Lw, Md, Wy.

STR Inbr (N): 30. Gen: *aabb*. Orig: Strong, to National Institutes of Health in 1951. Maintained by: N.

Swiss *See* SWR. *See* Z. Note also that extensive use is made of various lines of "Swiss" mice which are related to SWR and Z but are non-inbred.

SWR Syn: Swiss, Swiss-R, Swiss-8. Inbr (Ly): 43. Gen: *cc*. Orig: a strain of "Swiss" mice from A. de Coulon of Lausanne, Switzerland, inbred by Lynch. Charac: 44 per cent lung tumor, 19 per cent mammary tumor in breeding ♀ ♀ (3). Maintained by: He, Jax, Ly.

WG Inbr (Mi): 39+. Gen: *aaccpp*. Orig: Michie. Maintained by: Mi, Mo.

White label *See* WLL or WLO.

WLL Syn: White Label, White Label Leeds, Kreyberg albino. Inbr (Br): 29. Gen: albino. Orig: Kreyberg, to Bonser in 1935 (Kreyberg, *Brit. J. Cancer*, in press). *See* WLO. Charac: mammary cancer approx. 20 per cent in breeding ♀ ♀ (*J. Path. & Bact.*, **48**:263, 1939). Maintained by: A, Br, Ge*.

WLO Syn: White Label Oslo. Orig: Kreyberg, from same heterozygous female that gave rise to WLL stock. Charac: no mammary tumors for many years. Maintained by: Ge*, Kr.

YBL Syn: Y, yellows. Inbr (Rl): 41+. Gen: *A^{y}a*. Orig: Little (2). Charac: *A^{y}* (yellow) mice produced by outcross are obese, but there is no obesity in the inbred line. Maintained by: Re, Rr, Wy.

YBR/Rr Syn: Y, yellows. Inbr (Rr): 28+. Gen: *aabb*. Orig: Little, a branch of YBL (which was originally *Bb*) (*J. Exper. Med.*, **59**:229, 1934). Maintained by: Rr.

YBR/Wi Syn: Y, yellows. Inbr (Wi): 48. Gen: *A^{y}abb*. Orig: Little, via Heston. Maintained by: Ha, Wi.

Z Inbr (Gw): 39. Gen: *cc*. Orig: Gowen, from Swiss mice of the Yellow Fever Laboratory of the Rockefeller Inst. Charac: medium susceptibility to typhoid (*J. Infect. Dis.*, **77**:85, 1945). Maintained by: Gw.

Z *See* C3H.

ZR Syn: Zr/Chase. Inbr (Ch): 22. Gen: *aabbppss*. Maintained by: Ch.

ZRD Inbr (Rr): 20. Gen: *aabbddppssey-1ey-1ey-2ey-2*. Orig: Chase. Maintained by: Ch, Re, Rr.

3CAMG Inbr (St): 35. Gen: *aabb*, with blaze and belly streak. Orig: Strong, branch of BRS. Maintained by: St.

17 Syn: XVII. Gen: *cc*. Orig: Institut du Radium (5). Charac: low tumor. Maintained by: La.

50 Syn: L. Gen: *cc*. Orig: ♀XIX× ♂XLIII (5). Charac: 60–65 per cent mammary tumor. Maintained by: La.

101 Syn: 101BA$_g$. Inbr: 49 in 1948. Gen: +. Orig: Dunn. Maintained by: Rl.

129 Inbr (Rr): 12+, since reconstitution by crossing two sublines in 1948. Gen: *A^{w}A^{w}c^{ch}p/cp*. Orig: Dunn.

	Charac: useful for transplant and ova transfer studies. Maintained by: Am, Ha, Hu, Re, Rl, Rr.
1194	Inbr (Ly): 52. Gen: A^wA^w. Orig: Lynch, from Lathrop stock. Charac: low lung tumor, high mammary tumor (*J. Exper. Med.*, **43**:339, 1926; *Proc. Soc. Exper. Biol. & Med.*, **43**: 186, 1940). Maintained by: He, Ly.
XVII	*See* 17.
XXXIX	*See* CBA.
L	*See* 50.

APPENDIX 3

List of Abbreviations for Use in Symbolizing Substrains

A	Dr. O. Mühlbock, Antoni van Leeuwenhoek-Huis, Amsterdam C., Sarphatistraat 108, Holland.
Am	Dr. J. L. Ambrus, Department of Pharmacology and Biochemistry, Philadelphia College of Pharmacy and Science, Philadelphia 4, Pa.
An	Dr. H. B. Andervont, National Cancer Institute, Bethesda 14, Md.
Be	Dr. R. A. Beatty, Institute of Animal Genetics, West Mains Road, Edinburgh 9, Scotland.
Br	Dr. G. M. Bonser, Department of Experimental Pathology and Cancer Research, School of Medicine, Leeds 2, England.
Bs	Dr. P. Borges, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.
Bu	Dr. W. J. Burdette, Department of Surgery, Louisiana State University School of Medicine, New Orleans 12, La.
C or Cam	Professor R. A. Fisher, Department of Genetics, 44, Storey's Way, Cambridge, England.
Ca	Dr. T. C. Carter, Institute of Animal Genetics, West Mains Road, Edinburgh 9, Scotland.
Ch	Dr. H. B. Chase, Department of Biology, Brown University, Providence, R.I.
Ci	Dr. E. Caspari, Department of Biology, Wesleyan University, Middletown, Conn.
Co	Dr. L. C. Dunn and Dr. Salome Gluecksohn-Waelsch, Department of Zoölogy, Columbia University, New York 27, N.Y.
Cr	Professor J. Craigie, Imperial Cancer Research Fund, Burtonhole Lane, Mill Hill, London, N.W. 7., England.
Di	Miss M. Dickie, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.
Dm	Dr. L. Dmochowski, Medical School, Leeds 2, England.
Du	Dr. Wilhelmina Dunning, University of Miami, Coral Gables (University Branch), Fla.
Eh	Dr. J. Engelbreth-Holm, Universitetets patologisk-anatomiske Institut, Frederik V's Vej 11, Köbenhavn, Denmark.
Fa	Dr. D. S. Falconer, Institute of Animal Genetics, West Mains Road, Edinburgh 9, Scotland.
Fe	Miss E. Fekete, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.
Fl	Dr. J. L. Fuller, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.
Fr	Dr. F. Clarke Fraser, Department of Genetics, McGill University, Montreal, Canada.
Fs	Dr. E. Fredericson, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.
Fu	Dr. J. Furth, Division of Biology, Oak Ridge National Laboratory, Oak Ridge, Tenn.
G	Glaxo Laboratories, Ltd., Greenford, Middlesex, England.
Gd	Dr. H. D. Goodale, Williamstown, Mass.
Ge	Professor H. N. Green, Department of Pathology, The University, Sheffield 10, England.
Gl	Dr. A. Glücksmann, Strangeways Research Laboratory, Wort's Causeway, Cambridge, England.
Gn	Dr. E. L. Green, Department of Zoology, Ohio State University, Columbus 10, Ohio.
Go	Dr. P. A. Gorer, Department of Pathology, Guy's Hospital Medical School, London, S.E. 1., England.
Gr	Dr. H. Grüneberg, Department of Genetics, University College, Gower Street, London, W.C. 1., England.
Gs	Dr. L. Gross, Veterans Administration Hospital, 130 West Kingsbridge Road, Bronx 68, N.Y.

Gw	Dr. J. W. Gowen, Iowa State College, Ames, Iowa.
Ha	Dr. T. S. Hauschka, Institute for Cancer Research, Philadelphia 11, Pa.
He	Dr. W. E. Heston, National Cancer Institute, Bethesda 14, Md.
Hg	Frl. Professor Dr. P. Hertwig, Biologisches Institut, Universitätsplätz 7, Halle (Saale) Germany.
Ho	Mr. W. Hollander, Department of Genetics, Iowa State College, Ames, Iowa.
How	Dr. Alma Howard, Radiotherapeutic Research Unit, Hammersmith Hospital, Ducane Road, Shepherd's Bush, London, W. 12., England.
Hu	Dr. Katharine Hummel, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.
J or Jax	Inbred Nucleus of the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me. (under the supervision of Dr. E. S. Russell).
Ka	Dr. H. S. Kaplan, Department of Radiology, Stanford University School of Medicine, Clay and Webster Streets, San Francisco 15, Calif.
Ki	Dr. A. Kirschbaum, Department of Anatomy, University of Illinois College of Medicine, 1853 West Polk Street, Chicago 12, Ill.
Kr	Prof. L. Kreyberg, Universitetets Institutt for Patologi, Rikshospitalet, Oslo, Norway.
Ks	Dr. N. Kaliss, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.
L	Eli Lilly and Co., Indianapolis 6, Ind.
La	Dr. A. Lacassaigne, Laboratoire Pasteur, Institut du Radium, Rue d'Ulm, Paris, France.
Li	Dr. C. C. Little, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.
Lw	Dr. L. W. Law, National Cancer Institute, Bethesda 14, Md.
Ly	Dr. Clara Lynch, Rockefeller Institute for Medical Research, 66th Street and York Avenue, New York 21, N.Y.
M	Dr. J. H. Burchenal, Memorial Hospital and Sloan-Kettering Institute for Cancer Research, 410 East 68th Street, New York 21, N.Y.
Md	Dr. E. C. MacDowell, Department of Genetics, Carnegie Institution of Washington, Cold Spring Harbor, N.Y.
Mg	Mr. W. C. Morgan, Columbia University Biological Station, Nevis Estate, Irvington-on-Hudson, N.Y.
Mi	Dr. D. Michie, The University Museum, Oxford, England.
Mn	Dr. W. McNutt, The University of Texas, Medical Branch, Galveston, Tex.
Mo	Dr. R. H. Mole, Radiobiological Research Unit, Atomic Energy Research Establishment, Harwell, Didcot, Berkshire, England.
Mr	Dr. E. W. Miller, The J. H. Burn Research Laboratory, Royal Victoria Infirmary, Queen Victoria Road, Newcastle-upon-Tyne 1, England.
My	Dr. W. Murray, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.
N	Dr. G. Jay, Laboratory Aids Branch, National Institutes of Health, Bethesda 14, Md.
No	Dr. D. J. Nolte, Genetics Laboratory, Department of Zoölogy, University of of the Witwatersrand, Johannesburg, South Africa.
Or	Prof. J. W. Orr, Cancer Research Laboratory, Department of Pathology, The Medical School, Birmingham 15, England.
Pa	Dr. Edith Paterson, Christie Hospital and Holt Radium Institute, Manchester 20, England.
Pu	Dr. B. D. Pullinger, Research Department, Glasgow Royal Cancer Hospital, Hill Street, Garnethill, Glasgow C. 3., Scotland.
Re	Dr. Elizabeth Russell, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.
Rl	Drs. W. and Liane Russell, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn.
Ro	Frl. U. Roth, Hamburg 36, Jungiusstr. 6, Zoologisches Institut, Germany.
Rr	Dr. M. Runner Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.
Ru	Dr. H. P. Rusch, McArdle Memorial Laboratory, University of Wisconsin, Madison 6, Wis.
Sc	Dr. J. P. Scott, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.
Si	Dr. Jean B. Siri, Division of Medical Physics, Donner Laboratory, Univer-

sity of California, Berkeley 4, Calif.

Sk Dr. H. E. Skipper, Southern Research Institute, 917 South Twentieth Street, Birmingham 5, Ala.

Sn Dr. G. D. Snell, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

Sp Dr. W. L. Simpson, Detroit Institute for Cancer Research, 4811 John R Street, Detroit 1, Mich.

St Dr. L. C. Strong, Department of Anatomy, Yale University School of Medicine, 333 Cedar Street, New Haven 11, Conn.

T Dr. L. Butler, Department of Zoölogy, University of Toronto, Toronto 5, Canada.

Tn Dr. A. Tannenbaum, Department of Cancer Research, Medical Research Institute, Michael Reese Hospital, 29th and Ellis Avenue, Chicago 16, Ill.

Vi Miss Emelia Vicari, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

Wa Mr. S. G. Warner, Biological Laboratory, Springville, N.Y.

Wi Prof. J. W. Wilson, Department of Biology, Brown University, Providence 12, R.I.

Wo Dr. D. L. Woodhouse, Cancer Research Laboratory, Department of Pathology, The Medical School, Birmingham 15, England.

Wy Dr. G. W. Woolley, Sloan-Kettering Institute for Cancer Research, 410 East 68th Street, New York 21, N.Y.

Announcements

The New York Cancer Society, Inc.

The formation of a new clinical society designated as The New York Cancer Society, Inc., was announced at a dinner meeting of the Founders' Group at the Yale Club of New York City, on Friday, March 28th. With an initial composition of 36 specialists interested primarily in the diagnosis and treatment of neoplastic diseases, the object of the society is to encourage research and to exchange information bearing on this subject.

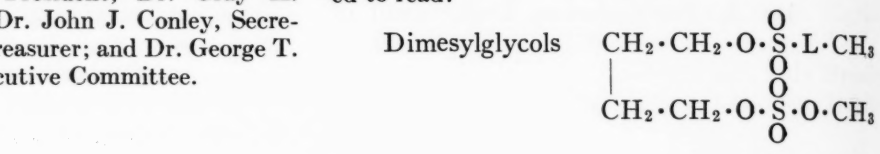
Officers elected by the society for the coming year were: Dr. Frank Glenn, President; Dr. Gray H. Twombly, Vice-President; Dr. John J. Conley, Secretary; Dr. William Harris, Treasurer; and Dr. George T. Pack, Chairman of the Executive Committee.

Errata

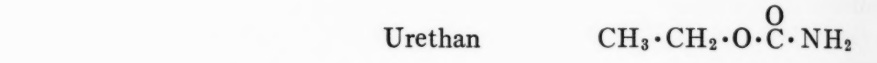
The article entitled "Studies on Urinary Thiocyanate Excretion from Administered Nalononitriles" by E. M. Gal, E. Swirnofsky, and D. M. Greenberg, in Vol. **12**: 263, 1952, line 7, should read: ". . . thiocyanate in the controls was 132γ per day against 89γ for the tumor-bearing rats."

In the article entitled "Mechanism of Carcinogenesis: *A Review*" by Boyland, **12**:79, 1952, the formulae for the dimesylglycols and for urethan should be amended to read:

Dimesylglycols



Urethan



Further Plans for Publication of Negative Data on Compounds Tested in Experimental Cancer Chemotherapy Programs

In the March, 1952, issue of *Cancer Research*, pages 241–42, a new service was announced and inaugurated, namely, the publication of data on negative results obtained in the tests of compounds for their effects upon neoplastic growth. This program will be continued in the future on the basis warranted by submission of new data; however, there exists such a back-log of negative data that they cannot be published promptly in regular issues of the journal. Therefore, before the end of 1952 there will be published a supplement number of *Cancer Research* devoted entirely to the presentation of negative data. The supplement will be edited by C. Chester Stock, Sloan-Kettering Institute for Cancer Research, with the assistance of Joseph Leiter, National Cancer Institute, and Arthur Furst, University of San Francisco.

Data for inclusion in the supplement number are now invited. It is requested that they be submitted by Oct. 15, 1952, for inclusion in the supplement. Any material received after that date will be considered for publication in tables in future supplements. Abbreviations may be used when they follow the form in the March, 1952, publication. All material should be sent to Dr. C. Chester Stock, 444 East 68th Street, New York 21, N.Y., in whose office it will be typed for duplication by photo-offset process. The material is to be presented in tabular form as follows:

Compound name	Compound source	Physical constant of sample used	Tumor	Age of tumor or days after transplant	Host species, strain	No. of deaths/ No. of test animals	Dose mg/kg/day or per cent in food	Av. wt. change; treated/ controls	No. of treatments or duration of feeding	Route of administration	Vehicle	Remarks

It is realized that all the above information may not be available from all studies. Data not complete by the above standard will be acceptable, although the editorial committee will reserve the right to decide in any case where there is a question of the adequacy of the data. A brief description of the experimental procedure used, the method of evaluation, and sample positive data, if available, are requested for data submitted for publication in the supplement number.